RISK ASSESSMENT OF A XENOBIOTIC IN RAT

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DEPARTMENT OF ZOOLOGY, FACULTY OF SCIENCE

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CERTIFICATE

This is to certify that the thesis "**RISK ASSESSMENT OF A XENOBIOTIC IN RAT**" incorporate the results of investigation carried out by the candidate herself and analyzed in the Department of Zoology, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara.

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(Hetal Roy)

Guiding Teacher
(Dr. B. Suresh)

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Just as a coconut tree bears the weight of coconuts on its head and gives nectarine water throughout its life in return for a little water that was given to it during the first year; in the same way a person should never forget the help that one had received.

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LIST OF ABBREVIATIONS

AChE - Acetylcholiesterase

ALP- Alkaline phosphates

ALT- Alanine transaminase

AO – Acridine orange

AST- Aspartate transaminase

CAs- Chromosome aberration

CDNB - 1-chloro 2,4 dinitrobenzene

CE - Cholesterol Esterase

CO - Cholesterol Oxidase

DTNB- 5,5-dithiobis-(2-nitrrobenzoic acid)

EB- Ethidium bromide

FOB – Functional observation battery

FST- Forced swimming test

fT3 - free Triiodothyronine

fT4 - free thyroxine

GGT- Gamma glutamyl transpeptidase or Gamma glutamyltransferase

GPx – Glutathine peroxidase

GST- glutathione s-transferase

HDL-C- High density lipoprotein cholesterol

HSDA - Sodium N-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline

IC_{50} – Inhibition concentration

LD_{50} – Median lethal dose

LDH – Lactate dehydrogenase

LDL-C – Low density lipoprotein cholesterol

LMA – Low melting agarose

LPO- Lipid peroxidase

MDA - Malondialdehyde

MN- Micronucleus

MTT- (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)

nAChR - nicotine acetylcholine receptor

NCE - Normochromatic erythrocyte

NO – Nitric oxide

NTE - Neuropathy target esterase

PBS - Phosphate buffer saline

PCE - Polychromatic erythrocyte

POD - Peroxidase from Horseradish

SOD - Superoxide dismutase

TCA - Trichloroacetic acid

TEM – Transmission electron microscopy

TPO - Thyroperoxidase or thyroid peroxidase

TSH - thyroid stimulating hormone

INTRODUCTION

Many industrial organic products today are composed of molecular structures that are different from natural substrates or the metabolic intermediates of natural substrates. The un-natural characteristics make those man-made organic chemicals foreign to the biosphere and thus the organics are called xenobiotics (Nyuk and Huang, 2012).

Xenobiotics are encountered by humans on a daily basis and include drugs, environmental pollutants, cosmetics, pesticides and even components of the diet. These chemicals undergo metabolism and detoxication to produce numerous metabolites, some of which have the potential of causing inadvertent effects such as toxicity. They can also block the action of enzymes or receptors used for endogenous metabolism or affect the efficacy and/or bioavailability of a co-administered drug (Johnson *et al*., 2012).

Pesticides, one of the known xenobiots, are a widely used, structurally diverse group of chemicals designed to kill or repel pests such as insects, weeds, or microbes, but they can also have potential toxic effects on human health. Humans are exposed to pesticides via inhalation, dermal contact and ingestion due to occupational exposure, in the environment and through contaminated food (Gilden *et al*., 2010; Abassa *et al*., 2012). Pesticides are suspected to have harmful neurological, immunological, reproductive and cancerous health effects. Pesticides are categorized into four main types, based on their substituent chemicals: herbicides, fungicides, insecticides and bactericides (Gilden *et al*., 2010).

An insecticide is a class of pesticide used against insects. They include ovicides and larvicides used against the eggs and larvae of insects respectively. Insecticides are used in agriculture, medicine, industry and households. The use of insecticides is believed to be one of the major factors behind the increase in agricultural productivity in the 20th century (Van Emden and Pealall, 1996). Nearly all insecticides have the potential to significantly alter ecosystems; many are toxic to humans; and others are concentrated in the food chain. Insecticides fall into two categories – inorganic and organic. The organic insecticides are further divided into different groups such as Organophosphorus compounds (OP), Organochlorine compounds (OC),

Carbamates (C), Pyrethrins/Synthetic Pyrethroids (SP), Neonicotinoids, Insect Growth Regulators (IGR), etc. (Van Emden and Pealall, 1996).

Neonicotinoids are a class of neuroactive insecticides chemically related to nicotine. Nicotine is the main biologically active alkaloid found in tobacco extract and has been used for many years as an insecticide. Nicotine consumption for insecticidal use exceeded 2,500 tons worldwide after the Second World War and then gradually declined to less than 200 tons by the early 1980s as a broad range of more selective, effective and cheaper insecticides became established (Ujvary, 1999; Rose, 2012). Although nicotine was not very effective as an insecticide and was toxic to mammals, research was undertaken in the 1970s and 1980s to discover potentially better insecticides based on the nicotine structure. The optimization of a lead compound in 1970s led to the synthesis of nithiazine, a potent nitromethylene with a thiazine ring; however, it was photolabile and thus not developed as an agricultural insecticide (Tomizawa and Casida, 2009). Further optimization resulted in the introduction of a chloropyridinylmethyl substituent and replacement of the nitromethylene group with nitroguanidine or cyanoamidine moiety to yield photostable molecule with similar potency (Tomizawa and Casida, 2009). This new class of systemic insecticides became known as neonicotinoids, based on their similarity to nicotine in terms of structure and action (Rose, 2012).

The development of this class of insecticides began with work in the 1980s by Shell and the 1990s by Bayer (Kollmeyer *et al*., 1999). The neonicotinoids are the only major new class of insecticides developed in the past three decades. The neonicotinoids were developed, mainly because they show reduced toxicity compared to the previously used organophosphate and carbamate insecticides. Most neonicotinoids show much lower toxicity in mammals than in insects, but some breakdown products are toxic (Chao and Casida, 1997). In the modern times, neonicotinoids are the most widely used insecticides in the world (Yamamoto, 1999). They are classified by the Environmental Protection Act 2002 as class II (WHO, 2009) and III toxins and are labelled with a signal word – "Warning" or "Caution" (Fishel, 2010).

The discovery of the neonicotinoid insecticides was an important milestone in the history of modern insect control. There are three types of neonicotinoid insecticides with chloropyridinylmethyl (imidacloprid or IMI, thiacloprid or THI, acetamiprid or ACE and nitenpyram or NIT), chlorothiazolylmethyl (thiamethoxam or TMX and clothianidin or CLO) or tetrahydrofuranylmethyl (dinotefuran or DIN) substituents (Ford, 2008). As the first major successful representative of this chemical class, Imidacloprid was introduced to the market in

1991, and since then, a series of six analogues has been launched [Thiacloprid (1985), Nitenpyram (1988), Acetamiprid (1989), Clothianidin (1989), Thiamethoxam (1992), and Dinotefuran (1994)]. The neonicotinoids are the fastest growing chemical class of insecticides, accounting for over 15% of the total insecticide market (Wollweber and Tietjen, 1999). Their tremendous success is based on their unique chemical and biological properties, such as broad-spectrum insecticidal activity, low application rates, excellent systemic characteristics, favourable safety profile and a new mode of action (Ford, 2008).

As the name suggests, the neonicotinoids are somewhat related to the alkaloid nicotine, primarily because they both act as potent agonists to nicotinic acetylcholine receptors (nAChRs). Neonicotinoids are thus neurotoxins and they bind selectively to the insect nAChR at a nanomolar range as compared to nicotine (micromolar range) (Kagabu and Medej, 1995).

Most neonicotinoids show low affinity for mammalian nicotinic acetylcholine receptors (nAChRs) while exhibiting high affinity for insect nAChRs (Tomizawa *et al*., 1999; Yamamoto, 1999). Mammals and insects have structural differences in nAChRs, that affect how strongly particular molecules bind, both in the composition of the receptor subunits and the structures of the receptors themselves (Tomizawa *et al*., 1999; Tomizawa, 2004). Nicotine, like the natural ligand acetylcholine, has a positively charged nitrogen (N) atom at physiological pH (Yamamoto, 1999; Tomizawa, 2004). A basic nitrogen will become positively charged in neutral aqueous solution because it is protonated by water. This positive charge gives these compounds a strong affinity to mammalian nAChRs. At the same time, the charge on nicotine lowers its effectiveness as an insecticide, because the blood–brain barrier prevents free access of ions to the central nervous system, and insect nAChRs are only present in the central nervous system (Yamamoto, 1999; Tomizawa, 2004). The blood–brain barrier does not prevent nicotine poisoning in mammals, because mammalian nAChRs are located in the peripheral nervous system and are necessary for vital functions such as breathing. The low mammalian toxicity of the neonicotinoid imidacloprid can be explained in large part by its lack of a charged nitrogen atom at physiological pH. The molecule shows weak affinity to mammalian nAChRs but strong affinity for insect nAChRs. Furthermore, the uncharged molecule can penetrate the insect blood–brain barrier, while the human blood–brain barrier filters it (Yamamoto, 1999). However, desnitro-imidacloprid, which is formed in a mammal's body during metabolism (Tomizawa, 2004) as well as by environmental breakdown (Svetlana, 2006), has a charged nitrogen and shows high affinity to mammalian nAChRs (Tomizawa,

2004). A study conducted on rats suggests that the neonicotinoids may adversely affect human health, especially the developing brain (Kimura-Kuroda *et al*., 2012).

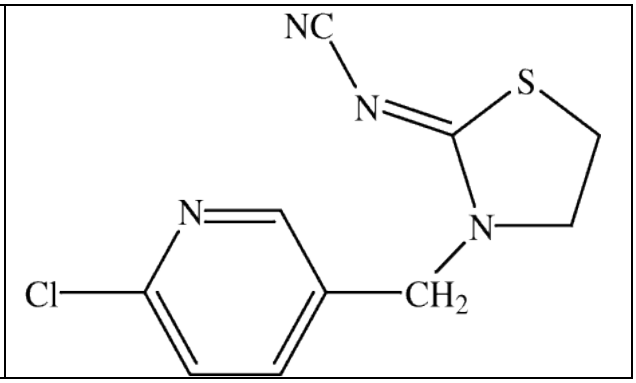
Thiacloprid [3-(6-chloro-3-pyridylmethyl)-1, 3-thiazolidin-2-ylidenecyanamide (IUPAC)], is an insecticide of the neonicotinoid class. It is effective against sucking insects on plants and companion animals, against turf insects and some beetles. Due to its systemic activity, thiacloprid is extensively used for soil application, seed and foliar treatment. Like the other neonicotinoids, thiacloprid shares structural similarity and a common mode of action with the tobacco toxin, nicotine (EPA, 2005).

Thiacloprid was registered as a neonicotinoid pesticide with the United States Environmental Protection Agency (USEPA). It possesses selective toxicity for insects relative to mammals and displays a broad spectrum of useful properties. These properties include high insecticidal potency, control of insects resistant to the major pesticides (e.g. organophosphates, carbamates and pyrethroids) and efficacy in soil application due to its mobility from the roots to the upper parts of plants (Kagabu *et al*., 2002). Ever since it was introduced in the insecticide market in 1985, the use of thiacloprid has increased yearly. For the most part, it is replacing the acetylcholinesterase inhibitors, the organophosphorus compounds and methylcarbamates (EPA, 2002).

Thiacloprid is marketed by Bayer CropScience Limited with trade names like Alanto, Bariard, Biscaya, Calypso, Monarca, and Proteus and with different formulation of suspension concentration (SC), Oil dispersion (OD), Granules (GR), Suspo-emulsion (SE) and Water dispersible granule (WG).

IDENTITY

Common name	Thiacloprid
Chemical name (IUPAC)	3-(6-chloro-3-pyridylmethyl)-1, 3-thiazolidin-2-ylidenecyanamide
Chemical name (CAS):	[3-[(6-chloro-3-pyridinyl)methyl]-2-thiazolidinylidene]- cyanamide
Manufacturer's code number	YRC 2894
CAS Registry number	111988-49-9
Molecular formula	C10H9ClN4S
Molecular mass	252.73 g/mol

Molecular structure	

Formulations

Formulation Content of active ingredients with Trade names

SC 480	480 g/L Thiacloprid Calypso
SC 240	240 g/L Thiacloprid Calypso
SC 240	240 g/L Thiacloprid Biscaya
SC240	240 g/L Thiacloprid Alanto

Chemical characteristics of technical thiacloprid

Colour	Yellowish
Physical State	Crystal powder
Odour	Odourless
Stability to normal and elevated temperatures, metals, and metal ions	Stable to elevated temperatures; stable in presence of metal and metal ions
Oxidation/reduction	Unaffected by reducing agents
Storage Stability	Stable for 2 weeks at 50°C
pH	7.40 at 20°C
Melting Point	136 °C
Relative density	1.46 g at 20°C
Dissociation constants in water	No basic or acidic properties in aqueous solutions
Water solubility; column elution method; shake flask method	In water = 185ug/L at 20°C
Vapor pressure	23 X 10-12 hPa at 20°C; 8 X 10-12 hPa at 25°C

Exposure

Pesticide applicators dealing with mixing, loading or applying thiacloprid insecticide as well as field workers entering freshly treated fields can come in direct contact with thiacloprid via skin contact or through inhalation of spray mists. Exposure to thiacloprid may also occur through

diet (food and water), when handling or applying the product or when picking fruits and vegetables. Thiacloprid enters the environment when used as an insecticide on pome fruit trees. Thiacloprid is not persistent in soil and is slightly persistent to persistent in water. The major transformation products formed in the soil are moderately persistent to persistent in this medium. The major transformation product formed in water is moderately persistent. Neither thiacloprid nor its major transformation products are expected to leach through the soil profile beyond 30 cm; therefore, they are not expected to enter groundwater. Based on its low volatility (vapour pressure and Henry's law constant), thiacloprid residues are not expected in the air (PMRA, 1998).

When assessing health risks, two key factors are considered: the levels where no health effects occur and the levels to which people may be exposed. The dose levels used to assess risks are established to protect the most sensitive human population (e.g. children and nursing mothers) (EPA, 2003). Aggregate dietary intake estimates (food and water) revealed that the general population and infants, the subpopulation that would ingest the most thiacloprid relative to body weight, are expected to be exposed to less than 6.2% of the acceptable daily intake (PMRA, 1998).

Metabolism

Thiacloprid is rapidly absorbed and is rapidly excreted after the following metabolic processes, with little remaining in the tissues. The metabolic processes were summarized as: 1) hydroxylation of the thiazolidine ring and subsequent glucuronidation (thiacloprid-amide {3-[(6-chloro-3-pyridinyl)methyl]-2-thiazolidinylidene}urea (M02)), 2) hydroxylation of the cyanamide moiety (6-CAN: 6-chloro-3-pyridinecarboxylic acid (M03)), 3) opening of the thiazolidine ring (thiacloprid-sulfoxide: N-[(6-chloro-3-pyridinyl)methyl]-N'-cyano-N-[2-(methylsulfinyl)- ethyl]urea (M08)), 4) formation of an oxazole ring (M09: N-{[6-(methylthio)-3-pyridinyl]-carbonyl}glycine), 5) oxidation and subsequent methylation of the thiazolidine ring (M12: Glucuronic acid conjugate of {3-[(6-chloro-3-pyridinyl)methyl]-4(or 5)-hydroxy-2-thiazolidinylidene} and 6) oxidative cleavage of the methylene bridge (thiacloprid sulfonic acid: Sodium 2-[[[(aminocarbonyl)amino]-carbonyl][(6-chloro-3-pyridinyl) methyl] amino] ethanesulfonate (M30)) (Figure 1). Only minor gender-related quantitative differences in metabolite profiles have been observed (EPA 2003; Zhang *et al*., 2012).

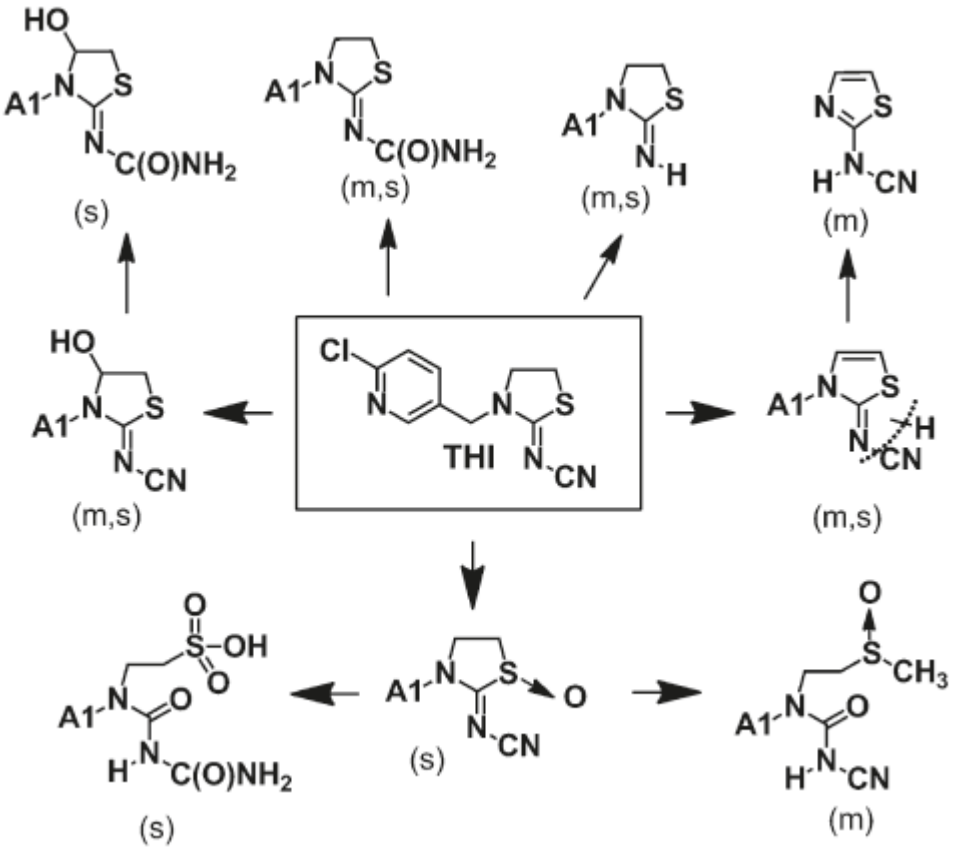


Figure 1: Biotransformation of thiacloprid in rodent (EPA, 2003)

Mechanism of action as a neurotoxicant

Thiacloprid as a neonicotinoid has a chloro-substituted heterocyclic group of chlorthiazolyl, joined to a second heterocyclic ring. An electron-withdrawing moiety of cyano group in thiacloprid is essential for insecticidal activity (Kagabu, 1999).

The toxicity of thiacloprid is based on interference of neurotransmission in the nicotinic cholinergic nervous system. Thiacloprid is specific for $\alpha_4\beta_2$ nAChRs of insect CNS. Thiacloprid binds to the nicotinic acetylcholine receptor (nAChR) at the neuronal and neuromuscular junctions in insects and vertebrates. The nAChR is an ion channel, whose endogenous agonist is the excitatory neurotransmitter acetylcholine (ACh). The receptor normally exists in a closed state; however, upon ACh binding, the complex opens a pore and becomes permeable for cations. The channel openings occur in short bursts, which represent the lifetime of the receptor-ligand complex. ACh is then rapidly degraded by the enzyme acetylcholinesterse (AChE). In contrast, thiacloprid bound to the nAChR is inactivated very slowly. Prolonged activation of the nAChR by thiacloprid causes desensitization and blocking

of the receptor and leads to paralysis and death (Matsuda *et al*., 2001; Matsuda *et al*., 2005; Tomizawa and Casida, 2005).

Toxicity

a. Insect

Neonicotinoid pesticides have been linked to the dramatic collapse in bee numbers over the last decade, which is why the UK Government banned their use in their country. Under practical circumstances, acetamiprid and thiacloprid can be as toxic to honey bees as other neonicotinoids. Thiacloprid toxicity has been found to be 150 fold more than other insecticides in a class of neonicotinoids (Iwasa *et al*., 2004; Mullin *et al*., 2010). Langer *et al*. (2010) found that thiacloprid is more sensitive for insect than tested physiological end points for the neurotoxic insecticide.

b. Mammal

Reports indicate that Thiacloprid induces moderate acute toxicity after oral (LD50, 396–836 mg/kg b.w.) and inhalation (LC50, 1.223 to > 2.535 mg/L) exposure in rats, with females being more sensitive than males. Thiacloprid has low acute dermal toxicity (LD50 > 2000 mg/kg bw) in rats. Both, the technical grade active ingredient thiacloprid and the end-use product of this insecticide, are known to induce health effects in animals when ingested and are considered to be potential skin sensitizers. Thiacloprid is not a skin irritant in rabbits and guinea pigs but it can cause slight eye irritation in rabbits (EPA, 2003; WHO, 2009).

Health effects in animals given daily doses of thiacloprid over long periods of time include effects on the liver, thyroid gland, adrenal gland, testes and prostate gland. It has been reported that when thiacloprid was given to pregnant animals, effects on the developing foetus were observed at doses that were toxic to the mother, indicating that the foetus is not more sensitive to thiacloprid than the adult animal. Effects on reproduction were seen at doses that were highly toxic to adult animals. Thiacloprid is not genotoxic at acute exposure but is known to cause cancer in animals. On exposure over a long period of time, it causes DNA damage by forming DNA adduct molecules and leads to cancer of liver, thyroid gland, ovary and uterine cancer in animals (PMRA, 1995; PMRA, 1996). Tumours occur by a non-genotoxic mechanism. Thiacloprid is known to induce ovary tumours in mice and uterine tumours in rats (EPA, 2003; WHO, 2009). Thiamethoxam, a neonicotinoid cynoderivative produces liver tumours in female and male mice (Green *et al*., 2005; Green *et al*., 2005a). In animal studies, moderate to high doses have resulted in CNS stimulation, similar to nicotine, which includes tremors, impaired papillary function, and hypothermia. There are also few indications that thiacloprid causes damage to the nervous system of adult animals, and signs of a structural

change in the brain have been observed in developing animals exposed before and after birth (WHO, 2009).

c. Human

It is unknown whether or not humans can be more susceptible than rodents or dogs. Thiacloprid formulated neonicotinoids are of relatively low toxicity to humans because they react less with human nicotinic receptor subtypes compared to insects, and they do not readily penetrate the human blood brain barrier. With poor penetration through the blood brain barrier, centrally mediated effects are not expected at low levels of exposure. Despite wide usage, reports of acute overdose or adverse effects after human exposure have been less. (PMRA, 1998). Nevertheless the following cases have been reported.

Hypotension and fatal ventricular dysrhythmias were reported in an adult who ingested 19.2 g of insecticide formulation. Weakness of the neck muscles and respiratory failure occurred in an adult after ingesting 50mL of a solution containing 17.8% thiacloprid. Respiratory failure and suspected pulmonary aspiration have been observed in a small number of adults following ingestion of thiacloprid alone. Coma and respiratory failure developed in an adult following intentional exposure of an unknown amount of an insecticide formulation; the patient recovered with supportive care. An adult developed severe agitation, respiratory failure, and disorientation following the inhalation of 17.8% thiacloprid, but recovered with supportive care. Thiacloprid is also likely to be Carcinogenic to Humans (NRA, 2001; EPA, 2006).

AIM OF THE STUDY

Neonicotinoid insecticides are a new class of agrochemicals and there is lack of systemic toxicity studies on thiacloprid pesticides. Besides its agricultural use, it is also used to control houseflies on poultry farms and to manage the pests of pet animals like cats and dogs. However, there is paucity of information available concerning the effects of thiacloprid on animal health (Tomizawa and Casida, 2005). The present predictive toxicology study was designed with an aim to elucidate the xenobiotic responses to thiacloprid by mammals - a non targeted organism. Such an assessment would give more information on the possible relationship between neonicotinoid pesticide application and systemic toxicity on a mammalian model.

In order to achieve the aim of the present study, i.e. to elucidate the thiacloprid induced systemic toxicity and toxicokinetics in mammals (using male Sprague Dawley (SD) rat as

the animal model), four parallel approaches, which could be treated as specific objectives were designed.

The first objective was to evaluate the hepatotoxic effects of thiacloprid in rat, following repeated oral administration. The liver is prone to xenobiotic-induced injury because of its central role in xenobiotic metabolism, its portal location within the circulation, and its anatomic and physiologic structure (Jones, 1996). The above objective was focused on finding whether the repeated administration of thiacloprid evoked hepatic impairment or not. For achieving this, whether a precise relationship exists between the occurrence of oxidative stress, peroxidation of membrane lipids, liver enzyme profile and histoarchitecture of liver of rat with repeated exposure of thiacloprid was investigated through the following studies a) Evaluation of biochemical profile (liver function test and estimation of biomolecules), b) Evaluation of oxidative stress enzymes and c) Histopathological evaluation of liver (Chapter 1).

The second objective was to assess thyroid toxicity after repeated oral administration of thiacloprid in SD rats. Considering the manufacturing details of the test compound and public summary of thiacloprid, there was clear indication that thiacloprid has potency to develop thyroid toxicity. However, the mechanism by which thiacloprid induces thyroid toxicity remains largely unknown. Thyroid hormone is important for maintaining physiological balance by controlling carbohydrate and lipid metabolism. Increased level of TSH promotes lack of adequate production of thyroid hormones which can lead to adverse effects on metabolism. One of the adverse effects is the development of limb ataxia due to thyrotoxicosis (Thompson and Potter, 2000). To evaluate thyroid toxicity via oral administration of thiacloprid, levels of T_3, T_4 and TSH hormone were estimated in serum of rat. Histoarchitectural changes in thyroid gland due to oral administration of thiacloprid were also studied. This was supplemented with lipid profile test of triglyceride, Cholesterol, HDL and LDL evaluation (Chapter 2) Attempt was also made to correlate thyrotoxicosis with liver function.

It is known that thiacloprid is a neurotoxic pesticide. Thiacloprid is specific for $\alpha_4\beta_2$ NAChR of insect CNS. Mammalian central nervous system has this specific type of nicotine acetyl choline receptor (Tomizawa and Casida, 2003). Thiacloprid bound to this receptor of CNS can possibly lead to exaggerated firing in neurons and development of ataxia. Hence, it was thought imperative to investigate any possible neurotoxic effects induced in mammals by thiacloprid. This was done by a series of studies such as Functional observation battery (FOB) test, estimation and histochemical localization of Acetylcholine esterase, estimation of Neurotoxic

esterase, estimation of oxidative stress enzymes, assessing the myelination pattern in different regions of brain and neurons, assessing viability of neurons and evaluation of histopathology of different regions of brain (Chapter 3).

Further, the evaluation of cytotoxicity gives idea about the potential toxicity of test substance. The predictive value of cytotoxicity tests is based on the idea of 'basal' cytotoxicity – that toxic chemicals affect basic functions of cells which are common to all cells, and that the toxicity can be measured by assessing cellular damage (Barile *et al*., 1994). Hence, thiacloprid induced cytotoxicity was evaluated through MTT assay, by observing morphological changes in bone marrow cells and assessing cell death (apoptosis or necrosis) in bone marrow cells. Also, genotoxic studies give indication about DNA damage, especially mutagenic or clastogenic effects of xenobiotics. Hence, in the present investigation, genotoxicity of thiacloprid was studied through the micronuclei test, chromosomal aberration test, DNA ladder assay and Single cell gel electrophoresis (SCGE) (Chapter 4).

MATERIAL AND METHODS

ANIMAL AND MAINTENANCE

Male Sprague Dawley (SD) rats procured from CPCSEA approved animal breeders were kept in the departmental animal house (827/ac/04/CPCSEA) at controlled temperature (24±2°C) and light-dark schedule (12:12) and were provided laboratory rat food (Pranav Agrochemicals) and water *ad libitum.* They were acclimatized for 10 days before starting the experimental procedure. Efforts were made to minimize the number of animals used and care was taken that they were subjected to minimal suffering. Dosing was performed on 7 weeks old rats weighing an approximate 225-275gms. All experimental protocols were approved by IAEC (Institutional Animal Ethics Committee) according to CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), India (Form B No. ZL/IAEC/13-2010). All the experiments were conducted in strict adherence to the procedures of the Drugs and Cosmetics rules 1945, Appendix - III animal care standard.

TEST CHEMICAL AND DOSAGE

A widely used formulated product, Thiacloprid (**Alanto 240, 21.7% SC**) manufactured by Bayer CropScience Ltd. India, was selected as the test chemical for the current study and was procured from the local market (Batch No. PGSC000002). Thiacloprid was diluted in distilled water for dose preparation according to desired concentration for oral exposure. Dose of thiacloprid was calculated based on the LD_{50} value given in the fact sheet of manufacturer (EPA, 2005). This was further validated in the current laboratory condition for Alanto 240, 21.7% SC through a dose range study. Subsequently, two doses (1/15 and 1/30 of median lethal dose) were selected as per standard regulatory norms for further repeated dose studies (OECD, 1998). Rats were divided into three groups *viz*., control group, low dose group and high dose group with 5 animals in each group. Treated groups of animals were given dosage for 28 days for subacute and 90 days for subchronic evaluation of thiacloprid toxicity. Rats were given oral exposure to thiacloprid once in a day at the concentration of 50mg/kg body weight for low dose (LTD) and 100mg/kg body weight for high dose (HTD) group. Every day food intake, morphological and behavioral changes were observed and recorded.

EXPERIMENTAL PROCEDURES

At the end of the treatment period, rats were kept for overnight fasting and sacrificed. For

neurotoxicity study, rats were perfused and brains were removed. Target organs of study were removed blotted free of blood or tissue fluid and weighed. Blood was collected from orbital sinus and serum was separated for evaluating various biochemical parameters. Tissues were weighed and homogenized in chilled PBS (pH 7.2) for various biochemical estimations.

1. Preparation of chemicals

Acetylthiocholine (ATC) solution
21.67mg of acetylthiocholine was dissolved in 1ml of distilled water.

Agarose
For comet assay: 1g agarose in 100ml of PBS
For electrophoresis: 1.4g in 100ml of TAE buffer

Carnoy's fixative
3:1 methanol: glacial acetic acid

Cholesterol Reaction Reagent
Horse radish peroxidase (1:50), Cholesterol oxidase (1:100) and Cholesterol esterase (1:200) were prepared in 10 ml of phosphate buffer (prepared freshly and used within 30 minutes).

0.1% Cresyl violet solution
Cresyl violet (0.1g) was dissolved in distilled water (100ml). 0.1ml of glacial acetic acid was added just before use and solution was filtered.

1-chloro-2, 4-dinitrobenzene (CDNB) reagent
30mM CDNB in 95% ethanol

Dichromate acetic acid reagent
Potassium dichromate (5%) and glacial acetic acid were mixed in the ratio of 1:3. From this 1ml was diluted with 4ml of acetic acid.

Direct bilirubin reagent
Sulfanilic acid 32mM was prepared with 0.01N hydrochloric acid.

5, 5-dithiobis-(2-nitrobenzoic acid) (DTNB) reagent
For AChE (Acetylocholinesterase) – 39.6mg of DTNB with 1mg $NaHCO_3$ was dissolved in 10ml of 0.1M phosphate buffer (pH 7).
For reduced glutathione - 40mg of DTNB was prepared in 100ml of 1% sodium citrate.

Folin-Ciocalteu reagent
5ml 2N Folin and ciocalteu's phenol reagent + 6ml distilled water (prepared freshly in amber coloured bottle).

GSH Standard solution
10mg of reduced glutathione was dissolved in 100ml distilled water

Geimsa Stain
3.8g of Giemsa powder was dissolved in 250ml of methanol, heated at 60ºC for 45 minutes in a water bath. This was stirred continuously, and then 250ml of glycerin was added to it. The stain was filtered and stored as the stock solution.

Incubation medium for AChE localization
5mg of substrate (i.e. acetylthiocholine iodide) was dissolved in 7.5ml of 0.1M phosphate buffer (pH 7.2). Then following items were added in order while stirring - 0.5ml of 0.1M Tri sodium citrate, 1 ml of 0.03M Copper sulfate and 1 ml of 0.005M Potassium ferricyanide 0.005M. (Total volume 10ml).

Low melting agarose (LMA)
1g of LMA was dissolved in 100ml of phosphate buffer.

Lowry solution
Solution A (alkaline solution) (500ml)
2.86g NaOH
14.31g Na_2CO_3

Solution B (100ml)
1.423g $CuSO_4.5(H_2O)$ in 100ml distilled water

Solution C (100ml)
2.853g Na_2 Tartarate.$2(H_2O)$ in 100ml distilled water
(Freshly prepared by mixing 50ml solution A+1ml of solution B + 1ml of solution C)

Lysis buffer (Comet assay)
2.5M NaCl (58.4g/mol), 100mM Tetra-sodium EDTA (416g/mol), 10mM Tris base (121.1g/mol), 1% sodium dodecyl sulfate. Solution diluted with ddH_2O while mixing to achieve a volume of 1L. The pH was adjusted to 10 and solution stored at room temperature.

Neutralization Buffer
1M Ammonium acetate (77.1g/mol) is prepared by dissolving 7.7g ammonium acetate in distilled water and made up to 100ml.

10% Neutral buffered formalin
Sodium phosphate monobasic 4.0g, Sodium phosphate dibasic 6.5g, 100ml Formaldehyde (37%) and made up to 1000 ml with distilled water.

Phosphate buffer
Stock solution A - 0.2M monobasic sodium phosphate monohydrate (27.6g/L).
Stock solution B - 0.2M dibasic sodium phosphate (28.4g/L).
51ml of A and 49ml of B solutions were mixed and diluted to a total volume of 200ml to make a 0.1M phosphate buffer of pH 6.8 at room temperature.

Phophate buffer saline (0.1M, pH 7.2)
8g of NaCl, 0.2g of KCl, 1.44g of $Na_2HPO_4 \cdot 2H_2O$ and 0.24g of KH_2PO_4 were dissolved in 1L of double distilled water. The pH was adjusted to 7.2 and solution was stored at 4ºC.

Phosphate solution (reduced glutathione)
0.3 M Na_2HPO_4 was dissolved in 1L of distilled water.

Precipitating reagent (reduced glutathione)
1.67g of metaphosphoric acid, 0.2g of EDTA disodium salt, 30g NaCl were dissolved in 1L of distilled water.

Pyrogallol solution
25.2mg of pyrogallol was dissolved in 1ml of 0.05M Tris-HCI buffer (pH 7.4) in an amber

colored vial. At the time of assay 0.5ml of this stock was diluted to 50ml with 0.05M Tris-HCl buffer (pH 7.4) to give 2mM solution

Reagent 1 (HDL-C)
α-cyclodextrin 0.5mM, dextran sulfate 0.5g/L, magnesium chloride 2mM, HSDA 0.3g/L, phosphate buffer pH 7.0 ± 0.1

Reagent 2 (HDL-C)
POD 15,000 U/L, PEG-CO 5,000U/L, PEG-CE 800 U/L, 4-aminoantipyrene 0.5g/L, phosphate buffer pH 7.0 ± 0.1, Triton X-100

Sodium azide solution
10mM is prepared by dissolving 6.5 mg NaN_3 in 1ml of distilled water.

Sodium nitrite reagent
Sodium nitrite 60mM was prepared in 25ml of phosphate buffer.

TAE buffer
4.84g of Tris base [tris (hydroxymethyl) aminomethane], 1.14ml of glacial acetic acid (17.4M) and 0.37g of EDTA, disodium salt were added to 1L of deionized water

2% Thiobarbituric acid (TBA) reagent
2g of TBA was dissolved in 100ml distilled water

Total bilirubin reagent
16mM Sulfanilic acid, 164mM hydrochloric acid, 4.4M dimethyl sulfoxide, 2.1mM caffeine were added in 50ml of phosphate buffer.

10% trichloro acetic acid (TCA)
10g of TCA was dissolved in 100ml of distilled water.

Unwinding/Electrophoresis Buffer (Comet assay)
0.3M NaOH (40.0g/mol), 10mM Tetra-sodium EDTA (416g/mol), 0.1% (w/v) 8-hydroxyquinoline, mixed with 700ml ddH_2O and 2% (v/v) DMSO (20ml) added while mixing. Once dissolved, additional ddH_2O was added to achieve a final volume of 1L. The pH was adjusted to 13.1 with concentrated NaOH or HCl.

2. Protocols for hepatotoxicity evaluation

2.1 Enzyme parameters

2.1.1. Estimation of Transaminase

Alanine aminotransferase (ALAT/ALT), formerly called Glutamic Pyruvic Transaminase (GPT) and Aspartate aminotransferase (ASAT/AST), formerly called Glutamic Oxalacetic Transaminase (GOT) are the most important representatives of a group of enzymes, the aminotransferases or transaminases, which catalyze the conversion of α-keto acids into amino acids by transfer of amino groups.

Alanine Transaminase (ALT) (E.C. 2.6.1.2)

The ALT level was estimated by the IFCC (International federation of clinical chemistry) (Bergmeyer and Horder, 1980) method. In the presence of alanine transaminase in the sample, L-alanine is converted to pyruvate. Pyruvate is reduced by lactate dehydrogenase to yield lactate with the oxidation of NADH to NAD. The ALT activity in the sample is proportional to the decrease in rate of absorbance of NADH which is measured at 340nm kinetically for 2 minutes.

$$\mathrm{L-Alanine} + \mathrm{2-Oxoglutarate} \xrightarrow{\mathrm{ALT}} \mathrm{L-Glutamate} + \mathrm{Pyruvate}$$

$$\mathrm{Pyruvate} + \mathrm{NADH} + \mathrm{H^+} \xrightarrow{\mathrm{LDH}} \mathrm{D-Lactate} + \mathrm{NAD^+}$$

0.1ml of sample and 1ml of freshly prepared reagent (L-alanine 500mM, α- ketoglutaric acid 15mM, LDH 10mM, NADH 0.18mM in 0.1M phosphate buffer pH 7.5) were mixed and incubated for 1 minute at room temperature. Optical density (OD) was measured at 340nm for 2 minutes at the interval for every 30 seconds and ΔA was calculated.

$$\text{Serum ALT activity(IU/L)} = \frac{\Delta A \times \text{Total volume of assay} \times 1000}{\text{Sample volume} \times 6.22}$$

Where, 6.22 = Millimolar absorptivity of NADH

Aspartate Transaminase (AST) (E.C. 2.6.1.1)

The AST level was estimated by the International federation of clinical chemistry (Bergmeyer *et al*., 1978) method. In the presence of AST in the sample, L-aspartate is converted to Oxaloacetate. Oxaloacetate is reduced by malate dehydrogenase to yield malate with the oxidation of NADH to NAD. The AST activity in the sample is proportional to the decrease in rate of absorbance of NADH which was measured at 340nm kinetically for 2 minutes.

$$\mathrm{L-Aspartate} + \mathrm{\alpha-Ketoglutarate} \xrightarrow{\mathrm{ALT}} \mathrm{Oxaloacetate} + \mathrm{L-Glutamate}$$

$$\mathrm{Oxaloacetate} + \mathrm{NADH} + \mathrm{H^+} \xrightarrow{\mathrm{MDH}} \mathrm{L-Malate} + \mathrm{NAD^+} + \mathrm{H_2O}$$

0.1ml of sample and 1ml of freshly prepared reagent (L-aspartate 500mM, Oxaloacetate 15mM, MDH 10mM and NADH 0.18mM in 0.1M phosphate buffer pH 7.5) were mixed and incubated for 1 minute at room temperature. OD was measured at 340nm for 2 minutes at the interval of every 30 seconds and ΔA was calculated.

$$\text{Serum AST activity(IU/L)} = \frac{\Delta A \times \text{Total volume of assay} \times 1000}{\text{Sample volume} \times 6.22}$$

Where, 6.22 = Millimolar absorptivity of NADH

2.1.2. Estimation of Alkaline Phosphatase (ALP) (E.C. 3.1.3.1)

Activity of alkaline phosphatase was estimated by the protocol of Tietz *et al*. (1983) approved by IFCC. ALP catalyzes the hydrolysis of phosphate esters bond in an alkaline environment (pH 10) and at an optimum temperature of 37°C, resulting in the formation of an organic radical and inorganic phosphate. p-Nitrophenyl Phosphate (PNPP) is a non-proteinaceous, non-specific substrate for alkaline phosphatases. p-nitrophenyl phosphate is hydrolyzed by ALP into p-nitrophenol, which is yellow at alkaline pH and its concentration can be measured at 405nm.

$$\mathrm{p-Nitrophenyl\,Phosphate} \xrightarrow{\mathrm{ALP}} \mathrm{p-Nitrophenol} + \mathrm{P}_i$$

0.3ml of buffered substrate (0.01M PNPP in 0.1M phosphate Buffer) and 0.1ml of sample were mixed and incubated for 15 minutes in water bath at 40°C. Then 0.5ml of 0.5N NaOH was added to develop colour and intensity of colour was measured at 405nm spectrophotometrically.

$$\text{Enzyme Activity} = \frac{\text{OD} \times \text{Conversion factor} \times \text{dilution} \times 100}{\text{Volume of Aliquote} \times \text{Tissue Weight}}$$

Unit - µ moles pNP released/15 minutes/mg tissue

2.1.3. Gamma Glutamyl Transpeptidase (GGT) (E.C. 2.3.2.2)

Calibrated method of Szasz (1969) was used to measure the activity of GGT. Gamma-glutamyl 3 carboxy-p-nitroanilide (GLUPA-c) and glycylglycine are converted by the action of GGT to p-nitroaniline and L-gamma glutamylglycine. The rate of increase in absorbance at 405nm due to the release of p-nitroaniline is directly proportional to the GGT activity.

$$\mathrm{L-gamma\,glutamyl\,3-carboxy\,4-nitroanilide} + \mathrm{Glycylglycine} \xrightarrow{\mathrm{GGT}} \mathrm{L-gamma\,glutamylglycylglycine} + \mathrm{5\,amino\,2\,nitrobenzoate}$$

1ml of regent buffer (glycylglycine 126mM, GLUPA-C 3.3mM and 0.095% sodium azide in Tris buffer (pH 8.1)) and 100µl of sample were mixed and incubated for 1 minute at room temperature. Absorbance of test was read first exactly at 60seconds and then, second, third, fourth readings were taken at an interval of 30 seconds at 405nm. The mean change in absorbance per minute (Δabs/min) was determined and results were calculated.

$$\text{Serum GGT activity(IU/L)} = \frac{(\text{A2} - \text{A1}) \times \text{Total volume of assay} \times 1000}{9.5 \times \text{Sample volume}}$$

Where, 9.5 = millimolar absorptivity of 5-amino-2-nitrobenzoate

2.1.4. Lactate Dehydrogenase (LDH) (E.C. 1.1.1.27)

Lactate dehydrogenase activity was measured according to the protocol of Buhl *et al*. (1977).

LDH catalyzes the oxidation of lactate to pyruvate accompanied by the simultaneous reduction of NAD to NADH. LDH activity in serum is proportional to the increase in absorbance due to the reduction of NAD at 340nm.

$$\text{Lactate} + \text{NAD} \xrightarrow{\text{LDH}} \text{Pyruvate} + \text{NADH}$$

50µl of sample was mixed with 1ml of reagent buffer containing 55mM L-lactate and 5.8mM NAD in Tris buffer (pH 8.9). OD was recorded after 30 seconds (A_1) and again noted exactly after 90 seconds (A_2) at 340nm. The change in absorbance (A_2-A_1) multiplied by the factor 3376 (if volume of assay remains constant) which yield results in U/L.

$$\text{Serum LDH activity(IU/L)} = \frac{(\text{A2} - \text{A1}) \times \text{Total volume of assay} \times 1000}{6.22 \times \text{Sample volume}}$$

Where, 6.22 = Millimolar absorptivity of NADH

2.2. Estimation of Biomolecules

Total Protein

The blue colour developed by the reduction of the phosphomolybdic-phosphotungstic components in the Folin-Ciocalteu reagent by the amino acids tyrosine and tryptophan present in the protein plus the colour developed by the biuret reaction of the protein with the alkaline cupric tartrate are measured at 660nm in the method described by Lowry *et al*. (1951).

Standard graph of protein was prepared by 20mg/ml BSA. 0.5ml of Lowry reagent was mixed with sample and standard and then incubated for 15 minutes at room temperature. 0.2ml of Folin reagent was added to each test tube and incubated again at room temperature for 30 minutes in dark. OD was measured at 660nm against $CuSO_4$ blank.

Unit – mg protein/g of tissue weight or g/dl for serum /plasma

Glucose

Glucose was estimated by GOD/POD method (Glucose oxidase/Peroxidase) as described by Trinder (1969). Glucose oxidase oxidises the aldehyde group of specific substrate, β-D-glucose, to gluconic acid and generates hydrogen peroxide. Hydrogen peroxide thus produced is acted upon by peroxidase which transfers oxygen to the chromogen system, 4-aminoantipyrine and phenolic compound. The chromogen system gets oxidized to a red quinoneimine dye. The intensity of colour is directly proportional to the concentration of glucose and is measured photometrically at 505nm.

$$\text{Glucose} + \text{O}_2 \xrightarrow{\text{Glucose oxidase}} \text{Gluconate} + \text{H}_2\text{O}_2$$

$$H_2O_2 + \text{Phenolic Compound} + 4-\text{Aminoantipyrine} \xrightarrow{\text{Peroxidase}} \text{Coloured Complex} + H_2O$$

1ml reagent buffer (2000IU/L glucose peroxidase, 1200IU /L peroxidase and 0.246mM 4-amino antipyrene) was incubated at room temperature with 10µl of sample and standard. Absorbance was measured at 505nm against reagent blank.

$$\text{Glucose(mg/dl)} = \frac{\text{Absorbance of Sample}}{\text{Absorbance of Standard}} \times \text{Concentration of standard}$$

Unit = mg/dl in serum or mg/g of tissue

2.3. Estimation of Metabolite

Bilirubin

Total and direct bilirubin was estimated by the method described by Jendrassik and Grof (1938). Bilirubin is estimated by reacting it with diazotised sulfanilic acid obtained from sodium nitrite and sulfanilic acid solutions. Bilirubin when reacted with diazotised sulfanilic acid forms a pink coloured Azocompound. The unconjugated or free bilirubin takes longer time to react and requires caffeine as accelerator.

1ml of direct bilirubin reagent or 1ml of total bilirubin reagent was added to 100µl of sample or standard as well as blanks of respective studies. 100µl of Na nitrite reagent was mixed in all sample and standard tubes but not the blank tubes. OD was read at 555nm using water as a blank.

$$\text{Bilirubin(mg/dl)} = \frac{\text{Abs. of Unk.} - \text{Abs. of Blank}}{\text{Abs. of Std.} - \text{Abs. of Std. Blank}} \times \text{Concentration of standard}$$

3. Parameters for Neurotoxicity study

3.1. Acetylcholinesterase (AChE) (E.C. 3.1.1.7)

Acetylcholinesterase activity was evaluated as per the method described by Ellman *et al*. (1961). Activity of AChE was measured in whole blood, plasma, various regions of brain and neuroblastoma IMR cell line. The assay uses the thiol ester acetylthiocholine instead of the oxy ester acetylcholine. AChE hydrolyses the acetylthiocholine to produce thiocholine and acetate. The thiocholine in turn reduces the DTNB liberating nitrobenzoate, which absorbs at 412nm.

$$\text{Acetylthiocholine} \xrightarrow{\text{AChE}} \text{Thiocholine} + \text{acetate}$$

$$\text{Thiocholine} + \text{dithiobisnitrobenzoate(DTNB)} \longrightarrow \text{Yellow color}$$

For blood AChE activity

200µl of blood was mixed with 5ml of DTNB reagent. 2ml of this was used for estimating whole blood enzyme activity and 3ml was centrifuged at 2000rpm for 10 minutes at 10ºC for plasma AChE activity. In each tube 0.1ml of acetylthiocholine substrate was added and mixed thoroughly. Change in absorbance was recorded for a period of 5 minutes at an interval of 1 minute at 412nm.

$$R = \frac{\Delta A \times \text{Total volume of assay} \times 1000 \times 5.74 \times 10^{-4}}{\text{Sample volume}}$$

Where, R = Rate in micromoles of acetylthiocholine hydrolyzed /minute / dl

For brain AChE activity

The rats ware decapitated; brains were removed quickly and placed in ice-cold PBS. Various regions of brain were quickly dissected out on chilled petri dish placed on crushed ice. The tissues were weighed and homogenized in 0.01% Triton X-100 in 0.1M phosphate buffer (pH 8). 0.4ml of aliquot of homogenate was added in tube containing 100µl of DTNB and contents were mixed properly. In each tube 0.1ml of acetylthiocholine substrate was added and mixed thoroughly. Change in absorbance was recorded for a period of 5 minutes at an interval of 1 minute at 412nm.

$$R = \frac{\Delta A \times \text{Total volume of assay} \times \text{dilution factor} \times 5.74 \times 10^{-4}}{\text{Weight of tissue} \times \text{Sample volume}}$$

Where, R = Rate in micromoles of acetylthiocholine hydrolyzed /minute / g tissue weight

For cell line AChE activity

After removing the medium from the neuroblastoma cells, the cells were washed three times with Phosphate buffered saline (PBS). The cultured cells were extracted by adding 4ml of High ionic strength buffer (10mM $NaHPO_4$, pH 7.5, 1M NaCl, 10% Triton X-100 and 1mM EDTA) and incubated for 5 minutes at room temperature. Cells were then centrifuged at 12,000rpm in a 15 ml tube for 20 minutes. Supernatant was removed. 20µl of supernatant was mixed with 50µl of DTNB. 10µl of acetylthiocholine was added and change of absorbance was measured for 2 minutes at 30 seconds time interval at 412nm.

3.2. Neurotoxic Esterase (NTE) (E.C. 3.1.1.5)

The procedure is a modification for rat brain of a standard method for hen brain (Johnson, 1977) and for IMR cell line (Correll and Ehrich, 1991). Brain homogenate (50µl) was added to Tris-EDTA buffer (950µl). Phenyl valerate was introduced in 0.03% Triton X-100 in Tris-

EDTA buffer (1ml) and incubated for 15 min. The reaction was stopped with 1% SDS and 0.025% 4-aminoantipyrine in distilled water (1ml). Addition of 0.04% potassium ferricyanide in water (0.5 ml) allowed spectrophotometric determination at 490nm of phenol liberated by NTE.

$$\text{NTE activity} = \frac{\text{OD} \times \text{Volume of assay} \times \text{dilution factor} \times 1000}{\text{Weight of tissue} \times \text{Sample Volume}}$$

Unit – mM phenol liberated/15minutes/g of tissue weight

3.3. Localization of enzyme

Acetylcholine esterase

Direct colouring method described by Karnovsky and Roots (1964) was adapted for the localization of acetylcholine esterase. 15µm thick cryosections of various regions of brain were taken. Sections were incubated at 37ºC for 2 hours in the incubating medium for AChE localization and then rinsed with distilled water. Slides were observed under microscope (Leica DM2500) at10X for localization of AChE and pictures captured using EC3 Camera (utilizing LEICA LAS EZ (V 1.6.0) software).

3.4. Functional observation battery (FOB)

Forced swimming test

According to the protocol described by Cryan *et al*. (2002), rats were placed in glass cylinder containing water. The water level in the glasses was high enough to prevent the rat from touching the bottom of the cylinder with its paws or tail, and low enough to avoid an escape through the top opening of the cylinder. The rats were thus forced to swim in the cylinders for 10 minutes. This activity was repeated every month. Parameters like active swimming, floating and dipping were recorded with time.

Rotarod Test

The rotarod test is used to assess motor coordination and balance in rodents. This is done through examining the balance of the rat on a rotating rod (rotarod). One can investigate brain injury or neuromuscular damage if the rat fails to keep its balance on the rotarod. The method described by Hamm *et al*. (1994) was used to assess the motor activity of experimental animals. Time (latency) taken by the rat to fall off the rotating rod at different speeds or under continuous acceleration (e.g. from 4 to 40rpm) was recorded. Each animal was observed for 5 minutes and each fall was noted.

3.5. Nissl body stain

Nissl substance (rough endoplasmic reticulum of neuron) is lost after cell injury and if the axon degenerates, the myelin covering also breaks down. Nissl body staining method was used for the detection of neuronal damage. 15µm thick cryosections of brain were stained with warmed (50ºC in oven) 0.1% cresyl violet for 8 to 10 minutes and rinsed immediately with distilled water. Differentiation was done with 95% ethyl alcohol for 2-10 minutes and sections were checked under the microscope (Leica DM2500) at 10X magnification for examining Nissl body which are stained pink-violet.

4. Analysis of Biochemical parameters for Thyroid toxicity study

4.1. Estimation of biomolecules

Cholesterol

Cholesterol concentration in serum was estimated by method given by Allain *et al*. (1974). The cholesterol esters are hydrolysed to free cholesterol by cholesterol esterase (CE). The free cholesterol is then oxidised by cholesterol oxidase (CO) to cholesterol 4-en-3-one with the simultaneous production of hydrogen peroxide. The hydrogen peroxide reacts with 4-aminoantipyrine and phenolic compound in the presence of peroxidase to yield a coloured complex which is read at 505nm. The intensity of colour produced is directly proportional to the concentration of total cholesterol in the sample.

$$\text{Cholesterol ester} \xrightarrow{\text{Cholesterol Esterase}} \text{Cholesterol} + \text{free fatty acid}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol Oxidase}} \text{Cholesterol} - 4 - \text{en} - 3 - \text{one} + H_2O_2$$

$$2H_2O_2 + 4 - \text{aminoantipyrene} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine dye} + H_2O$$

1ml of cholesterol reaction reagent was mixed with 10µl of standard (10mg/ml) and test sample. 50µl of 16mM 4-aminoantipyrene was added to it and then incubated for 30 minutes at room temperature. OD was measured at 505nm against reagent blank.

$$\text{Cholesterol (mg/dl)} = \frac{\text{OD of sample} \times \text{Volume of assay} \times \text{Conc. of Std} \times 100}{\text{OD of standard} \times \text{Volume of Sample}}$$

HDL Cholesterol (HDL-C)

Serum HDL-C levels were estimated using a kit (Reckon Diagnostics Pvt. Ltd., India) based on the method described by Grundy *et al*. (1993). In this method, pretreatment or centrifugation steps are not required. The method involves a two-reagent format. The first reagent contains α-cyclodextrin and dextran sulphate to stabilize LDL, VLDL, and chylomicrons. The second

reagent contains PEG modified enzymes that selectively react with the cholesterol present in the HDL particles. Consequently, only the HDL cholesterol is subject to cholesterol measurement at 690nm.

10µl of serum sample or standard were incubated with 300µl reagent 1 for 10 minutes at room temperature. 100µl of reagent 2 was added to it and again incubated for 10 minutes. Absorption was measured at 690nm.

$$\text{HDL} - \text{C Concentration (mg/dl)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times \text{Calibrator Concentration}$$

LDL- Cholesterol (LDL-C)

The LDL cholesterol assay was also done using a kit (Reckon Diagnostics Pvt. Ltd., India) based on the method described by Grundy *et al*. (1993) for measuring LDL cholesterol levels from serum or plasma. This kit uses a specific detergent formulation to selectively dissolve non-LDL lipoprotein particles (HDL, VLDL and chylomicrons) while leaving LDL particles intact. The dissolved cholesterol is degraded by the cholesterol esterase and cholesterol oxidase enzymes. Subsequently a second detergent is added to the sample to solubilize the remaining LDL particles. The soluble cholesterol and cholesterol esters are oxidized by cholesterol oxidase to produce hydrogen peroxide. The hydrogen peroxide product then reacts with bis (4-sulfobutyl)-m-toluidine and 4-aminoantipyrine to form a coloured product. The resulting colour change is measured at 550nm and is proportional to the amount of LDL cholesterol originally present in the sample.

10µl of serum sample or standard were incubated with 750µl reagent 1 for 10 minutes at room temperature. 250µl of reagent 2 was added to it and again incubated for 10 minutes. Absorption was measured at 550nm

$$\text{LDL} - \text{C Concentration (mg/dl)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times \text{Calibrator Concentration}$$

Triglyceride

The method given by Fossati and Lorenzo (1982) was adopted for triglyceride estimation in serum. Lipase hydrolyses triglycerides sequentially to Di and Monoglycerides and finally to glycerol. Glycerol Kinase (GK) using ATP as PO_4 source converts Glycerol liberated to Glycerol-3-Phosphate (G-3-Phosphate). G-3-Phosphate Oxidase (GPO) oxidises G-3-Phosphate and forms Dihydroxy acetone phosphate and hydrogen peroxide. Peroxidase (POD) uses the hydrogen peroxide formed, to oxidise 4- Aminoantipyrine and TOOS (N-ethyl-N-Sulphohydroxy propyl-m Toluidine) to a purple coloured complex. The absorbance of the

coloured complex is measured at 546nm (530-570nm or with yellow filter) which is proportional to Triglyceride concentration.

$$\text{Triglyceride} + H_2O \xrightarrow{\text{Lipase}} \text{Glycerol} + \text{fatty acid}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{Glycerol kinase}} \text{Glycerol} - 3 - \text{phosphate} + \text{ADP}$$

$$\text{Glycerol} - 3 - \text{phosphate} + O_2 \xrightarrow{\text{GPO}} \text{Dihydroxyacetone phosphate} + H_2O_2$$

$$2H_2O_2 + 4\ \text{aminoantipyrene} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine dye} + H_2O$$

10µl of standard (10mg/ml) or sample was mixed with freshly prepared triglyceride reaction reagent and incubated at 37ºC for 15 minutes. 50 µl of 16mM 4-aminoantipyrene was added to the mixture and absorbance was taken at 550nm against reagent blank.

$$\text{Triglyceride (mg/dl)} = \frac{\text{OD of sample} \times \text{Volume of assay} \times \text{Conc. of Std} \times 100}{\text{OD of standard} \times \text{Volume of Sample}}$$

4.2. Assessment of Thyroid hormone and TSH

For the quantitative determination of thyroid stimulating hormone (TSH), Triiodothyronine (T_3) and thyroxin in serum, ELISA kit of TSH, T_3 and T_4 was used (GenWay Biotech Inc., USA). The kit is based on the principle of a solid phase enzyme-linked immunosorbent assay. The assay system utilizes a unique monoclonal antibody directed against a distinct antigenic determinant on the intact TSH, T_3 and T_4 molecules. Mouse monoclonal anti-TSH, anti T_3 and anti T_4 antibody was used for solid phase immobilization (microtiter wells), and goat anti-TSH, anti T_3 and anti T_4 antibody was present in the antibody-enzyme (horseradish peroxidase) conjugate solution. The test sample was allowed to react simultaneously with the two antibodies, resulting in the TSH, T_3 and T_4 molecules being sandwiched between the solid phase and enzyme-linked antibodies. After a 60-minute or overnight incubation at room temperature, the solid phase was washed with water to remove unbound labelled antibodies. A solution of 3,3',5,5'-Tetramethylbenzidine (TMB) was added and incubated for 20 minutes, resulting in the development of a blue colour. The colour development was stopped with the addition of 1N HCl, and the resulting yellow colour was measured at 450nm with a microplate reader (Metertech Σ960). The concentration of TSH, T_3 and T_4 is directly proportional to the colour intensity of the test sample.

5. Parameters for Genotoxicity Study

5.1. Micronucleus (MN) assay

Incidence of micronuclei serves as an index of genetic damage. A micronucleus is formed during the metaphase or anaphase transition of mitosis or meiosis (cell division). MN test is a

rapid and reliable assay for genotoxic assessment of test substance. MN test was performed according to prescribed protocol of Romagna and Staniforth (1989).

Blood was collected in 2ml EDTA coated vacutainer and blood smear was prepared on a glass slide and fixed with methanol. Immediately after sacrifice of rat, femur bone was dissected out and then bone marrow was removed by injection of RPMI 1460 medium. The collected cells were centrifuged at 3000rpm for 5 minutes. Pellet of bone marrow cells was resuspended in RPMI medium, of which a small drop was smeared on glass slide and fixed in absolute methanol. The smear was air dried and stained for 5 minutes with 10µg/ml acridine orange, a fluorescent dye and then washed with PBS twice. Slides were observed under fluorescent microscope (Leica DM2500) to score micronucleus. 1000 cells were counted per rat.

5.2. Polychromatic erythrocyte (PCE)/Normochromatic erythrocyte (NCE)

Bone marrow smear was stained with 2% Geimsa stain for 5 minutes and then washed with phosphate buffer. 200 bone marrow cells were scored for PCE (immature or intermediate erythrocyte with ribosome) and NCE (mature erythrocyte without ribosome). PCE/NCE ratio was calculated for analysis of cytotoxicity of test chemical. Micronucleated PCE (MNPCE) and micronucleated NCE (MNNCE) were also scored with Geimsa staining.

5.3. Chromosomal aberration

Standard method for preparation of metaphase chromosome was followed (Evans *et al*., 1964). Rats were weighed and colchicine (4mg/Kg body weight) was administered intraperitoneally 2½ hrs before harvesting bone marrow cells. Immediately after sacrifice of rat, femur bone was dissected out and then bone marrow was removed by injection of RPMI 1460 medium. The collected cells were centrifuged at 3000rpm for 5 minutes. Pellet of bone marrow cells was resuspended in 0.6% KCl and then agitated for a minute. Sample was incubated at 50ºC in water bath for 45 minutes and centrifuged for 10 minutes at 3000rpm. Methanol:Glacial acetic acid fixative (3:1) was added to the pellet and agitated properly for a minute and then centrifuged at 3000rpm for 10 minutes to collect the pellet. This step was repeated twice. Cells were spread on a chilled glass slide which was then kept on a hot plate at 60ºC for 10 seconds. Finally slides were stained with 2% Geimsa for 10 minutes

5.4. Comet Assay

Comet assay was performed in rat bone marrow cells, according to the *in vivo* comet assay guidelines of Tice *et al*. (2000), as described by Saquib *et al*. (2009). Briefly, 100µl bone marrow cell suspensions of rats were mixed with 100µl of 1% Low Melting Agarose (LMA)

(in PBS). The cell suspension (100µl) was then layered onto frosted slides, pre-coated with agarose (1% in PBS) and kept at 4°C for 10 min. After gelling, a layer of 100µl of LMA (1% in PBS) was added. Cells were lysed overnight in a lysis buffer and then rinsed in PBS twice before subjecting to DNA denaturation in cold electrophoretic buffer at 4°C for 20 minutes. Electrophoresis was performed at 25V (300mA) at 4°C for 30 minutes. Slides were then washed three times with neutralization buffer. Each slide was stained with 20µg/ml ethidium bromide solution for 2 minutes. Slides were analyzed at 40X magnification (excitation wavelength of 515-560nm and emission wavelength of 590nm) using a fluorescence microscope (Leica DM2500). Images from 100 cells (50 from each replicate slide) were randomly selected and subjected to image analysis with CometScore software (TriTek Corporation, Virginia). Mean values of the Olive tail movement (OTM), tail length (µm) and tail intensity (%) were separately analyzed for statistical significance. To quantify the DNA damage, tail length (TL) and tail movement (TM) were evaluated. Tail length (length of DNA migration) is related directly to the DNA fragment size and presented in micrometres. It was calculated from the centre of the cell. Tail movement was calculated as the product of the tail length and the fraction of DNA in the comet tail.

5.5. DNA ladder assay

DNA fragmentation in the form of a ladder due to the endonucleolytic attack is reportedly considered as a characteristic of apoptosis (Basnakian and James, 1994). Bone marrow cells were lysed in lysis buffer with 10% sodium dodecyl sulfate (SDS) and 50µg/ml proteinase K for 2 hrs at 45°C. The cell lysates were first extracted with Tris-saturated phenol, phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v), then with chloroform–isoamyl alcohol (24:1), precipitated overnight at -20°C in two volumes of absolute ethanol in the presence of 0.3M Na acetate, and recovered by centrifugation. Pellets were air-dried, resuspended in TAE buffer. Electrophoresis was carried out in a 1.4% agarose gel containing 5µg/ml ethidium bromide. The gel was examined and photographed under a Gel Documentation system to visualize intra-nucleosomal DNA fragmentation (laddering), a characteristic of apoptosis or smearing of DNA due to necrosis.

5.6. Assessment of cell morphology for apoptosis

Bone marrow smear was fixed followed by staining with acridine orange:ethidium bromide (1:1) solution, slides were incubated for 2 minutes with the stain and washed with PBS twice. At least 1000 bone marrow cells of each group were examined under fluorescent microscope (Leica DM2500) using a fluorescein filter and 40X objective. Green fluorescence is observed

for viable cells and red fluorescence for necrotic cells, while the apoptotic cells show red and green fluorescence with apoptotic characteristics.

6. Oxidative stress parameters

6.1. Lipid Peroxidation product (Malondialdehyde (MDA))

The lipid peroxidation product present in the tissues was estimated by thiobarbituric acid (TBA) method (Janero, 1998). Malondialdehyde (MDA) is a low-molecular-weight end product formed via the decomposition of certain primary and secondary lipid peroxidation products. At low pH and high temperature, MDA readily participates in nucleophilic addition reaction with 2-thiobarbituric acid (TBA), generating a red fluorescent MDA-TBA adduct that absorbs at 532nm.

To 0.2ml of tissue homogenate, 0.2ml of 8% SDS, 1ml of 20% acetic acid and 1ml of 2% TBA were added. The mixture was made up to 4ml with phosphate buffer and then incubated in a water bath at 95°C for 60 minutes. After cooling, 3ml of 10% TCA was added and shaken vigorously. After centrifugation at 3000rpm for 10 minutes, the supernatant was taken and its absorbance was read at 532nm. The level of lipid peroxidase was expressed as nanomoles of MDA released/ hour/g tissue.

$$\text{MDA liberated} = \frac{\text{OD of sample} \times \text{dilution factor} \times 10^9 \text{ moles}}{\text{E} \times \text{Tissue weight}}$$

E = Extinction coefficient of MDA (1.56×10^5)

6.2 Estimation of antioxidant enzymes

Catalase (E.C. 1.11.1.6)

Catalase activity was assayed by the method described by Sinha (1972). Dichromate in acetic acid is reduced to chromic acetate, when heated in presence of hydrogen peroxide with the formation of per chromic acid as an unstable intermediate. The chromic acetate formed is measured at 590nm. Catalase was allowed to split H_2O_2 for different periods of time. The reaction was stopped at different time intervals by the addition of dichromate acetic acid mixture and the remaining H_2O_2 was determined by measuring chromic acetate spectrophotometrically after heating the reaction mixture.

The assay mixture consisted of 0.5ml of tissue homogenate, 0.5ml phosphate buffer (0.1M, pH 7.0), 0.5ml H_2O_2 (0.2M) in a final volume of 1.5ml. About 2ml dichromate acetic acid reagent was added in reaction mixture at the interval of 15, 30, and 45 and 60 seconds, incubated in boiling water bath for 10 minutes then cooled. Absorbance was recorded at 590nm.

$$\text{Catalase activity} = \frac{\text{OD of sample} \times \text{total volume of assay}}{\text{Volume of sample} \times \text{Tissue weight}} \times \frac{1}{\text{mg protein concentration}}$$

Unit = µmole H_2O_2 liberated/ minute/ mg protein

Superoxide dismutase (E.C. 1.15.1.1)

The activity of superoxide dismutase (SOD) was assessed by method described by Marklund and Marklund (1974). Superoxide anion is involved in auto-oxidation of pyrogallol at alkaline pH. The SOD inhibits the auto-oxidation of pyrogallol, which can be determined as an increase in absorbance per two minutes at 420nm on a spectrophotometer. The SOD activity was measured as unit/ml of homogenate. One unit of SOD is defined as the amount of enzyme required to cause 50% inhibition of pyrogallol auto-oxidation.

The reaction mixture for auto-oxidation consisted of 2ml of Tris-HCl buffer, 0.5ml of 2mM pyrogallol and 1.5ml water. Initially, the rate of autoxidation of pyrogallol was noted at an interval of 30 seconds for two minutes. The assay mixture for the enzyme contained 2ml of 0.05M Tris-HCI buffer, 0.5ml pyrogallol, aliquots of the homogenate and water to give a final volume of 4ml**.** The rate of inhibition of pyrogallol auto-oxidation after the addition of the enzyme was noted.

The enzyme activity was expressed in terms of units/mg protein in which one unit corresponds to the amount of enzyme that inhibited the autoxidation reaction by 50%.

Unit = % inhibition of pyragallol/min/mg tissue

Glutathione peroxidase (GPx, E.C. 1.11.1.9)

The activity of glutathione peroxidase in the samples was determined by the method of Rotruck *et al*. (1973). Enzyme preparation was allowed to react with H_2O_2 in presence of GSH for a specific time period. The GSH content remaining after the reaction was measured by the method of Ellman *et al*. (1961).

The reaction mixture in a total volume of 1ml contained 0.2ml of phosphate buffer, 0.2ml EDTA, 0.1ml sodium azide and 0.5ml of the enzyme source (tissue homogenate/ plasma/ hemolysate). 0.2ml of glutathione and 0.1ml of H_2O_2 were added to reaction mixture and incubated at 37°C for 10 minutes. The reaction was arrested by the addition of 0.5ml of 10% TCA. The tubes were centrifuged and the supernatant was assayed for GSH by adding phosphate buffer and Ellman's reagent (DTNB) into the supernatant. A blank was treated similarly to which 0.2ml of enzyme was added after incubation and absorbance was recorded at 412nm. The activity of glutathione peroxidase was expressed as mM of GSH consumed/ mg tissue.

$$\text{GPx activity} = \frac{\text{OD of sample} \times \text{volume of assay} \times \text{dilution}}{\text{CF} \times \text{Volume of sample} \times \text{Tissue weight}}$$

Where, CF = 0.00373

Glutathione-S-transferase (GST, E.C. 2.5.1.18)

The method described by Habig *et al*. (1974) was adopted for estimation of GST activity which was measured by following the increase in absorbance at 340nm using 1-chloro 2,4 dinitrobenzene (CDNB) as a substrate.

1ml of phosphate buffer, 0.1ml of CDNB and 1ml of tissue homogenate were mixed. The volume was adjusted to 2.9ml with water. The reaction mixture was pre-incubated at 37ºC for 5 minutes and the reaction started by the addition of 0.1ml of 30mM glutathione. The absorbance was recorded for 5 minutes at 340nm. A system devoid of enzyme served as the blank. The specific activity of GST was expressed as μmoles of CDNB- GSH conjugate formed/ minute/ mg protein).

$$\text{GST activity} = \frac{\text{OD of sample} \times \text{volume of assay}}{9.6 \times \text{Volume of sample} \times \text{mg of protein}}$$

Where 9.6 = mM extinction coefficient of CDNB-GSH conjugate

6.3. Reduced glutathione (Non-Enzymatic antioxidants)

The reduced glutathione level was determined by the method of Beutler *et al*. (1963). This method was based on the development of yellow colour when thiol reagent, 5,5'- dithio-bis-2-nitrobenzoic (DTNB) reacts with GSH present in tissue sample forming 5-thio nitrobenzoic acid (TNB) and GS-TNB, which can be measured at 412nm. The level of TNB and GS-TNB is equivalent to the GSH present in the tissue.

0.2ml of sample was mixed with 0.8ml of EDTA solution. To this 2ml of precipitating reagent was added, mixed thoroughly and kept for 5 minutes before centrifugation. Tubes were centrifuged at 3000rpm for 10 minutes. 1ml of the filtrate, 1ml of 0.3 M phosphate solution and 1ml of DTNB reagent were added and the colour developed was read at 412nm with a spectrophotometer. A set of standard solutions containing 20-100μg of reduced glutathione was treated similarly.

$$\text{GSH concentration } (\square\text{g/g tissue}) = \frac{\text{OD of sample} \times \text{volume of assay} \times \text{dilution}}{\text{Volume of sample} \times \text{tissue weight}}$$

7. Histopathology

Rats were sacrificed and target organs were removed and washed with PBS. Tissues were cut and fixed in 10% neutral buffered formalin. Tissue samples were dehydrated in upgrading alcohol series (50%, 70%, 90% and 100%; 2hours in each). Xylene was used as a clearing agent. Paraffin wax was used as the embedding medium. Tissues were processed for 2.5hrs in paraffin: xylene series (25:75, 50:50 and 75:25) and then finally kept in 100% paraffin. Paraffin block was prepared and tissue sections were taken using a microtome.

Tissue section was deparaffinized in xylene and rehydrated in down grade of alcohol series. Haematoxylin was used to stain nuclei of cells for 5 to 10 minutes. Differentiation was done by dipping the slide in ammonia solution, followed by a wash with water. Sections were covered with eosin for cytoplasmic staining. Slides were rinsed and dehydrated in increasing concentrations of alcohol, then xylene and mounted in DPX. Slides were observed under the microscope (Leica DM2500) for analysis of histopathological changes.

8. Transmission electron microscopy (TEM)

Method described by Glauert (1974) was adopted for TEM. Tissues were cut into pieces of 2-3mm size and fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer for 24 hrs at 4ºC. The tissues were rinsed with buffer and osmicated in 2% osmium tetroxide. Tissues were rinsed again and dehydrated using increasing alcohol concentrations, followed by rinsing in propylene oxide twice for 15 minutes on the rotator for each rinse. The specimen was put in a mixture of 50% propylene oxide and 50% resin for at least 2 hours and then in 100% resin as embedding medium. Resin blocks were prepared. Sections were cut on ultra microtome with glass knife and collected on gold grid. Sections were stained in uranyl acetate for 20 minutes and then rinsed. The sections were exposed to NaOH for 10 minutes and again rinsed. Sections were observed under a transmission electron microscope.

9. *In vitro* study

IMR 32, a neuroblastoma cell line was procured from the National Chemical Laboratory, Cell culture facility, Pune. Culture was maintained as an adherent cell line in T25 flask by providing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 100µl of antimycotic solution at 37°C in a 5% CO_2:95% air-humidified atmosphere. All the cell culture solutions were purchased from HiMedia Chemicals Ltd., USA. Cells were passaged as needed using 0.5% trypsin-EDTA.

Cell Viability test

Cells were exposed to 0.5% trypsin EDTA solution for 3 to 5 minutes and reaction was stopped by adding chilled phosphate buffer. Cells were collected in 15ml centrifuge tube and centrifuged for 10 minutes at 3000rpm. Cell pellet was resuspended in 1ml of phosphate buffer. 1 part of 0.4% trypan blue and 1 part of cell aliquot were taken and incubated for 2 minutes. Cells were observed under the microscope using hemocytometer. The unstained (viable) and stained (nonviable) cells were counted separately in the hemocytometer.

$$\% \text{ Cell Viability} = [\text{Total Viable cells (Unstained)}/\text{Total cells (Viable} + \text{Dead)}] \times 100$$

Viable Cells/ml = Average viable cell count per square x Dilution Factor x 10^4

Cytotoxicity test

The reduction of yellow 3-(4, 5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase occurs in viable cells. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. The cells are then solubilised with an organic solvent (eg. Isopropanol, DMSO) and the released, solubilised formazan reagent is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells the level of activity is a measure of the viability of the cells (Mosmann, 1983).

Cell viability was checked and equal number of cells were distributed in 96 well plate supplemented by 200µl DMEM medium with 10% FBS and 100µl antimycotic solution for 18 to 20 hrs. First line of wells was kept blank without the cells. The culture medium from each well was removed and cells were exposed to different concentrations of thiacloprid except for the second row of wells, into which 200µl of culture medium was added. Plate was incubated for different time intervals like 12 hrs and 24 hrs. Again culture medium with thiacloprid was removed and cells were washed with PBS. Cells were incubated with 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, HiMedia, USA) at a final concentration of 0.5 mg/ml in RPMI 1640 medium for 5 hours at room temperature in dark. After incubation, medium was removed and crystals of the insoluble formazan product were dissolved in 100µl of DMSO. The OD was read at 570nm using a microplate reader (Metertech Σ960). IC (inhibition concentration) 50 (IC50) value of thiacloprid was calculated.

$$\% \text{ inhibition } = 100 - \left(\frac{\text{OD of test}}{\text{OD of control}}\right) \times 100$$

Acridine orange/ Ethidium bromide (AO/EtBr) staining

Cells adhered on cover slips in 6-well plate after exposure to thiacloprid (12 hours) were stained with acridine orange and ethidium bromide stain for 60 seconds and observed under the fluorescent microscope (Leica DM2500). Live and dead cells can be identified easily with this stain on the basis of their membrane integrity. Dead cells show red fluorescence, live cells show green fluorescence and apoptotic cells show red and green fluorescence.

10. Statistical analyses

All quantitative variables are summarized and presented as mean and standard error around mean. In order to confirm the variances of the observations in the individual groups are equal Bartlett's test for homogeneity was done. The statistical significance of the differences between the mean values of control and experimental groups was evaluated through one-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test. Statistical analyses were performed using SPSS-PC Statistical Analysis Package (SPSS 12.0, SPSS Inc, Chicago, IL) and GraphPad Prism (version 6) software. Differences between groups were considered statistically significant at probability (p) ≤ 0.05.

CHAPTER 1

MECHANISTIC STUDIES ON HEPATOTOXICITY OF THE NEONICOTINOID THIACLOPRID 21.7% SC IN SD RATS

INTRODUCTION

Thiacloprid [3-(6-chloro-3-pyridylmethyl)-1,3-thiazolidin-2-ylidenecyanamide] is a member of the neonicotinoid insecticides registered for agricultural use by Bayer CropScience Ltd. (India) and is presently the most important commercial product because of its high efficacy against insects. It has outstanding potency and systemic action for crop protection against piercing-sucking pests, and is also highly effective for flea control on cats and dogs (Tomizawa and Casida, 2005).

Thiacloprid is reported selectively toxic to insects (Kagabu *et al*., 2002). All the neonicotinoids are synthesized by targeting the nicotinic acetylcholine receptor (nAChR) of crop insects. The neonicotinoids have unique physical and toxicological properties as compared with earlier classes of organic insecticides (Tomizawa and Casida, 2003). However, of the commercial neonicotinoids, acetamiprid, imidacloprid and thiacloprid are the most toxic to birds, and thiacloprid to fish. Several neonicotinoids are harmful to honeybees, either by direct contact or ingestion, but potential problems can be minimized or avoided by treating seeds and not spraying flowering crops (Kagabu and Akagi, 1997).

Upon uptake by mammals, most neonicotinoids undergo metabolic alterations at multiple sites but liver is a major site for metabolism of thiacloprid. The seven major commercial neonicotinoids are readily biodegraded by metabolic attack at their N-heterocyclylmethyl moiety, heterocyclic or acyclic spacer and N-nitroimine, nitromethylene, or N-cyanoimine tip. Phase I metabolism is largely dependent on microsomal cytochrome P450 (CYP) isozymes with *in situ* selectivity in hydroxylation, desaturation, dealkylation, sulfoxidation and nitro reduction. Cytosolic aldehyde oxidase is a nitroreductase for some neonicotinoids. Phase II metabolism involves methylation, acetylation and formation of glucuronide, glucoside, amino acid and sulfate- and glutathione-derived conjugates. Some neonicotinoids act as proinsecticides, which get metabolized to more potent nicotinic agonists (Casida, 2011). During detoxification, thiazolidine ring of thiacloprid is opened and the sulfur oxidized and

methylated (Klein, 2001). The chloro substituent is displaced presumably by glutathione, ultimately leading (via cysteine and -SH derivatives) to a methylsulfide. The nitrosoguanidine metabolite of neonicotinoids has moderate to high toxic potency (Tomizawa *et al.*, 2003), whereas the guanidine metabolite is highly activated against mammalian but deactivated against insect nAChRs (Chao and Casida, 1997; Tomizawa and Casida, 1999).

Cytochrome P450 is a superfamily of microsomal enzymes, which are found abundantly in the liver, gastrointestinal tract, lung and kidney, consisting of families and subfamilies of enzymes that are classified based on their amino acid sequence identities or similarities (Guengerich, 2003). Cytochrome P450 isozymes are involved in oxidative neonicotinoid metabolism especially the N-demethylated pathway (Schulz-Jander and Casida, 2002).

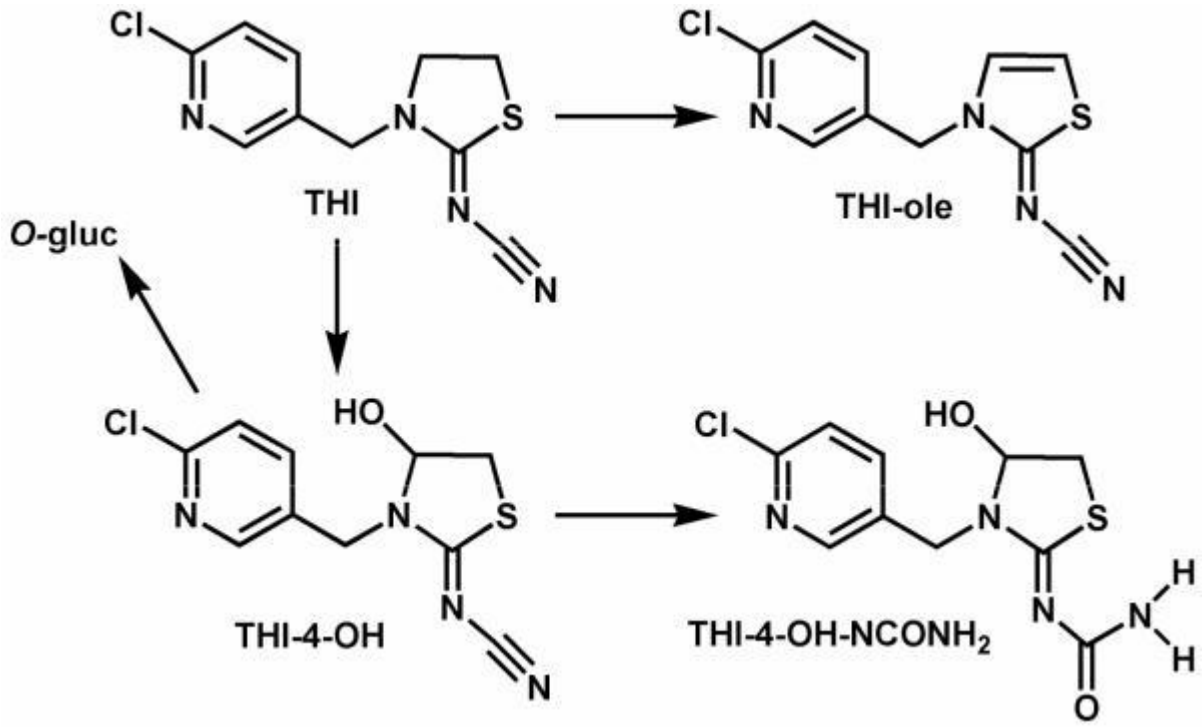


Figure A. Hydroxylation, hydration, glucuronidation, and desaturation of Thiacloprid (THI) *in vitro* and *in vivo* (adapted from Tomizawa and Casida, 2005).

Moreover, thiacloprid undergoes CYP dependent oxidative metabolism to the N-demethylated products. The chloro substituent is displaced to form the methylsulfide. With dinotefuran, hydroxylation of the tetrahydrofuran moiety leads to ring opening and liberation of an aldehyde that forms cyclic derivatives (Tomizawa and Casida, 2005). The C=N-NO2 (nitroguanidine) moiety is reduced to C=N-NO (nitrosoguanidine) and C=N-NH2 (aminoguanidine) and cleaved to the C=NH (guanidine) and C=O (urea) derivatives (Figure A) (Tomizawa and Casida, 2005). Oxidative metabolism generates reactive oxygen species which

are responsible for oxidative stress on liver. This may result in significant damage to cell structures (Devasagayam *et al.*, 2004).

It is also known that free radicals play an important role in the toxicity of pesticides and environmental chemicals (D'Almeida *et al*., 1997). Pesticide chemicals such as insecticides may induce oxidative stress leading to generation of free radicals and alterations in antioxidants or free radical scavenging enzyme systems (Kanbur *et al*., 2008; Duzguner and Erdogan, 2010). The data from experimental models either *in vivo or in vitro* indicate that the enzymes associated with antioxidant defense mechanisms are altered under the influence of pesticides (Thapar *et al*., 2002; Singh *et al.*, 2006). Moreover, oxidative stress and DNA damage have been proposed as mechanisms linking pesticide exposure to health effects such as cancer and neurological diseases. During the metabolism of insecticides, reactive oxygen species (ROS) and reactive nitrogen species (RNS) such as nitric oxide (NO) could be generated (Grisham *et al.*, 1999; Duzguner and Erdogan, 2012).

The elevated level of free radicals in liver leads to increased peroxidation of lipids and a decrease in the activities of antioxidant enzymes like superoxide dismutase (SOD), catalase and glutathione peroxidase. Low concentration of these enzymes facilitates the hepatocytic damage of liver, the major site of metabolism. Also, transaminase and alkaline phosphatase are important and critical enzymes in the liver and are responsible for detoxification processes (Rahman *et al*., 2000). Hence, any interference in levels of these enzymes leads to biochemical impairment and lesions of the tissue. Serum activity of liver enzymes like transaminase, alkaline phosphatase and /or glutamate dehydrogenase have been reported to be altered due to the chronic exposure to this neonicotinoid (Bhardwaj *et al*., 2010; Kammon *et al*., 2010). Imidacloprid, a neonicotinoid, is a hepatotoxic pesticide which causes necrosis or hypertrophy (Mohany *et al*., 2011). Another study reported that during short term oral exposure to imidacloprid for 14 and 28 days, serum total protein, albumin, globulin and creatinine levels remained normal (Balani *et al*., 2011).

Altered activity of antioxidant system of hepatocytes and liver marker enzymes also reportedly reflects as a pathological change in the histoarchitecture of liver, the major detoxifying organ (Amacher, 2002). Histopathological investigation of rat liver after 10 days of exposure to another neonicotinoid, thiamethoxam, also reportedly disturbed hepatic lobules, causing hydropic degeneration in the hepatocytes and also induced dilation of the hepatic sinusoids.

Thiamethoxam treated rats showed vacuoles in the cytoplasm of the hepatocytes. Some liver cells showed pyknotic or karyolytic nuclei (Shalaby *et al*., 2010). Sub-chronic exposure of thiacloprid on *Gallus domesticus* in the experiment of Goyal *et al*. (2010) induced degeneration of hepatocytes, fatty changes along with vacuolation and focal necrosis of hepatocytes.

Thiacloprid, the test chemical being assessed in the current study, belongs to the neonicotinoid class of insecticides. It mainly targets the nervous system of insects by mimicking the neurotransmitter receptor of insects (EPA, 2005). Few studies have been performed related to the toxic effects of neonicotinoids in mammals. Moreover, these studies have mostly focussed on the mechanism of neurotoxicity of neonicotinoids. However, many xenobiotics are capable of causing some degree of liver injury. The liver is prone to xenobiotic-induced injury because of its central role in xenobiotic metabolism, its portal location within the circulation, and its anatomic and physiologic structure (Jones, 1996). Few studies have demonstrated that acute exposure to neonicotinoid imidacloprid leads to oxidative and inflammatory effects in rats (Duzguner and Erdogan, 2010). Although thiacloprid is the most frequently used insecticide amongst the pesticides, such studies assessing toxic potential of thiacloprid in mammals are insufficient. Studies elucidating the chronic effects the test substance in non-target organisms such as mammals can be of significance since it throws light on the safety evaluation of the compound. The objective of the current study therefore, was to find whether repeated oral administration of thiacloprid has the potential to induce toxicity in a mammalian model, the rat, with the prime focus on the liver, a major detoxification site.

In the present study, we investigated the potential oxidative and chronic inflammatory effects of thiacloprid on the hepatic system of rats. The study was designed to reveal whether subacute and subchronic oral exposures of thiacloprid can develop liver toxicity in rat. To fulfil this objective, changes in liver enzymes like the transaminases (ALT and AST), alkaline phosphatase, γ-glutamyl transpeptidase and lactate dehydrogenase were estimated. The potential oxidative damage, generated via metabolism of thiacloprid in liver was also investigated by estimating activity of anti-oxidative enzymes and non-enzymatic antioxidants. Superoxide dismutase (SOD), glutathione peroxidase, glutathione-S-transferase, catalase and reduced glutathione activity were quantified during the study. Estimation of lipid peroxidation product was also one of the parameters observed to assess oxidative damage. The potential

relation between alterations in liver enzyme activity or antioxidant enzyme activity with histoarchitecture of liver was also explored.

MATERIAL AND METHODS

Thirty male Sprague Dawley (SD) rats weighing 250 ± 20gms were used for the present study. The animals were kept in clean cages in a well ventilated animal house of the Department of Zoology at The M.S. University of Baroda, Vadodara. All the protocols for experiments were approved by IAEC of the department of Zoology according to CPCSEA, India. Animals were exposed to a 12:12 light-dark schedule and were provided with standard rat feed (Pranav Agrochemicals, India) and RO water *ad libitum*. They were acclimatized for 10 days before starting the experimental procedure. Thiacloprid (Alanto 240, 21.7% SC) was diluted in physiological saline to obtain the desired dose concentrations. Animals were divided into following three groups of 5 animals each: Group 1, Control; Group 2, low dose [50mg/kg/body weight thiacloprid daily dose, (LTD)]; Group 3, High dose [100mg/Kg/body weight thiacloprid daily dose (HTD)] with 15 rats used for the 28 days study and 15 rats for the 90 days study. The rationale for selecting the said doses has been discussed elsewhere (Material and Methods). Food consumption was noted everyday and animals were weighed every week for analysis of body weight.

The animals were sacrificed 24 hours after the last drug administration. Before sacrifice, animals were subjected to overnight fasting and blood was collected from the orbital sinus of rats. They were sacrificed by cervical dislocation under mild diethyl ether anaesthesia. Liver was quickly excised from each rat, washed with ice-cold phosphate buffer saline, blotted free of fluids and used for biochemical studies. A part from the right lobe of liver was also fixed in 10% neutral buffered formalin for histopathological study. Blood was kept at 4°C for 2 hrs and serum was separated after centrifugation. Tissue samples were homogenized in ice cold phosphate buffer and centrifuged at 4°C on 3000rpm for 15 minutes. Supernatant was removed and used for biochemical studies.

Protocol I: Biochemical estimation

1. Assessment of enzyme activity

Alanine transaminase (ALT) (E.C. 2.6.1.2) was estimated by the method of the International federation of clinical chemistry (IFCC) (Bergmeyer and Horder, 1980). The ALT activity in

the sample is proportional to the decrease in rate of absorbance of NADH which is measured at 340nm kinetically for 2 minutes. Aspartate transaminase (AST) (E.C. 2.6.1.1) level was estimated by the method of the IFCC (Bergmeyer *et al*., 1978). The AST activity in the sample is proportional to the decrease in rate of absorbance of NADH which was measured at 340nm kinetically for 2 minutes.

Activity of alkaline phosphatase (ALP) (E.C. 3.1.3.1 was estimated by the protocol of Tietz (1983) approved by IFCC. The activity of the alkaline phosphatase was measured colourimetrically at 410nm. γ-glutamyl transpeptidase (GGT) (E.C. 2.3.2.2) activity was measured at 405nm according to calibrated method of Szasz (1969). Lactate dehydrogenase (LDH) (E.C. 1.1.1.27) activity was measured as per the protocol of Buhl *et al*. (1977) at 340nm.

2. Estimation of biomolecule

Total protein concentration was measured at 660nm according to the method of Lowry *et al*. (1951). Glucose level was estimated using GOD/POD (Trinder, 1969) method and absorbance was measured at 505nm.

Total bilirubin (metabolite) level in serum was measured using the method described by Jendrassik and Grof (1938). The intensity of colour produced in reaction is proportional to the concentration of bilirubin and was measured at 546nm.

Protocol 2: Oxidative stress parameters

The lipid peroxidation product present in the tissues was estimated by thiobarbituric acid (TBA) method (Janero, 1998). Red coloured complex of TBA reactive substance (TBRS) was measured at 532nm colourimetrically.

Catalase (E.C. 1.11.1.6) activity was assayed by the method of Sinha *et al*. (1972). Marklund and Marklund (1974) method was used for assessment of superoxide dismutase (SOD) (E.C. 1.15.1.1) activity. SOD inhibits the auto oxidation of pyragallol in a rate limiting fashion which was assessed at 420nm. The activity of glutathione peroxidase (GPx, E.C. 1.11.1.9) in sample was determined by the method of Rotruck *et al*. (1973). Enzyme preparation was allowed to react with H_2O_2 in presence of GSH for a specific time period. The GSH content remaining after the reaction was measured by the method of Ellman *et al*. (1961).

Habig *et al*. (1994) method was adopted for estimation of glutathione-S-transferase (GST, E.C. 2.5.1.18) activity which was measured by following the increase in absorbance at 340nm using 1-Chloro-2,4-dinitrobenzene (CDNB) as a substrate.

The level of reduced glutathione, a non enzymatic antioxidant, was determined by the method of Beutler *et al.* (1963). This method is based on the development of yellow colour when thiol reagent, 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) reacts with GSH present in tissue sample forming 5-Thionitrobenzoic acid (TNB) and GS-TNB, which can be measured at 412nm. The level of TNB and GS-TNB is equivalent to the GSH present in the tissue.

Protocol 3: Histopathological Examination

After overnight fasting, rats were sacrificed and livers were dissected out immediately. For the histopathological evaluation of liver, tissue was first fixed in 10% neutral buffered formalin solution for 24 hours and dehydrated in an upgrading ethanol series. Xylene was used as clearing agent before embedding in paraffin wax. Paraffin blocks of tissues were sectioned using a microtome, rehydrated and then stained with haematoxylin and eosin. Sections were dehydrated again, following which they were mounted in DPX. Slides were observed under the microscope (Leica DM2500) for analysis.

Statistical analysis

Statistical analysis was carried out using SPSS 12.0 for Windows and GraphPad Prism (version 6). The significance of the differences between mean values was evaluated through one-way ANOVA. Post hoc comparisons were carried out using the Bonferroni test. The data are presented as mean ± SEM. The minimum level of significance was set at $p \leq 0.05$.

RESULTS

In the current oral exposure study, rats were subjected to 50 and 100mg/kg body weight of thiacloprid (daily) and conditions such as bradypnea, laboured breathing, tremors, reduced motility and nose bleeding were observed in these animals during the period of exposure. After 26 days of thiacloprid exposure, clear ptosis was observed in rats receiving high dose and after 40 days, ptosis was common in both the treatment groups. Thiacloprid exposure for one month reduced the hind leg movement and it was observed that animals developed a drag during movement. This observation was common for both the treatment groups after 40 days of exposure to the test compound. Also, nasal secretion was commonly observed. Piloerection of hair situated around the eyes was observed routinely after exposure. Further, during subchronic exposure to thiacloprid, two rats from high dose group showed signs of extreme intoxication

viz., difficulty in locomotion, excessively laboured breathing, and were also unable to consume food and water after 78 days and had to be sacrificed early.

The weekly food intake of control and experimental groups of rats is given in Table 1.1. Food consumption in treatment groups was slightly lower during the toxicity period of evaluation of thiacloprid in the 28 days study as compared to control group (Figure 1.1). Values in Table 1.2 show significantly lower food consumption by rats during the 90 days treatment period, both for the low dose ($p \leq 0.05$) and high dose ($p \leq 0.01$) groups (Figure 1.2).

The mean alterations in body weight of rats are summarized in Table 1.3. For animals of both high and low dose treatment groups, a decrease in body weight was observed during the 28 days study, but values were not significantly different from those of control animals. However, significant decrease in the body weight of both low dose ($p \leq 0.05$) and high dose ($p \leq 0.01$) treatment groups of animals was observed during the subchronic study.

At the end of the drug exposure period, rats were sacrificed and observations on gross anatomy were also made. 'Milk spots' described as white patched lesions of necrosis (Biehl, 1984) were commonly observed in animals of the high dose treatment group during both the studies and also in animals of high dose group during subacute study. Also, a change in spleen colour and necrosis at its edges was observed in high dose group. White cysts of necrosis were also observed. Table 1.3 shows liver weight at the end of treatment schedule for the subacute and subchronic studies. Weight of liver was found to be increased significantly ($p \leq 0.05$) during subchronic exposure at 100mg/kg body weight as compared to that of control group of rats (Figure 1.3).

The effect of repeated 28 day exposure to thiacloprid at doses of 50mg/kg and 100mg/kg/body weight on serum enzyme parameters is shown in Table1.4 and Figure 1.4. Alanine aminotransferase activity increased significantly ($p \leq 0.05$) in serum of high dose treated rats as compared to controls. However, difference in ALT activity between low dose and control group was marginal and was not statistically significant during this treatment regime. Aspartate transaminase activity increased insignificantly in serum of both groups of treated rats as compared to control rats. It was observed that exposure to both 50mg/kg and 100mg/kg body weight of thiacloprid caused significant increase in alkaline phosphatase activity. Level of significance was low ($p \leq 0.05$) for LTD and high ($p \leq 0.01$) for HTD group of rats. Neither

dose group of thiacloprid treatment showed any significant change on γ-glutamate transpeptidase (GGT) activity. Lactate dehydrogenase activity was not significantly altered in low dose group but was seen significantly increased ($p \leq 0.05$) in high dose treatment group as compared to values of control group of animals.

Activities of various liver marker enzymes following subchronic thiacloprid exposure are shown in Table 1.5 and Figure 1.5. The obtained results indicate that 1/15 and 1/30 of median lethal dose of thiacloprid induced significant increase in ALT activity ($p \leq 0.05$) for both low and high dose groups ($p \leq 0.001$) of rats. Activity of ALT was observed to be significantly ($p \leq 0.05$) higher in HTD as compared to low dose treatment group. AST activity was found to be increased for the low dose group as compared to control but the difference was not found statistically significant. AST activity was observed to be significantly higher ($p \leq 0.05$) in 100mg/kg body weight thiacloprid treatment group. Oral administration of test chemical induced hepatic damage by altering the activity of ALP. Significantly higher ALP activity was observed in both low dose ($p \leq 0.05$) and high dose ($p \leq 0.001$) groups as compared to control group. GGT activity in serum was observed higher in both the treated groups of rats as compared to control group of animals. In both the treatment groups, significant increase in level of LDH enzyme activity was also observed. As compared to control group, the activity of LDH was higher at $p \leq 0.05$ for LTD and $p \leq 0.01$ for HTD rats.

Mean alterations in biomolecule concentration in serum for both subacute and subchronic studies are summarized in Table 1.6 and 1.7 respectively. At the end of 28 days of thiacloprid exposure, there was no significant difference in total serum protein concentration between the treatment groups of rats. Even though the treated animals showed marginal hike in serum protein concentration compared to controls they were statistically not significant. Protein concentration in serum was significantly higher ($p \leq 0.05$) after subchronic exposure to thiacloprid in both the treatment groups as compared to control group of rats (Figure 1.6). Glucose concentration was observed to be drastically low in both the low dose ($p \leq 0.01$) and high dose ($p \leq 0.001$) groups after subacute oral exposure to thiacloprid (Figure 1.7). Glucose concentration decreased significantly ($p \leq 0.001$) in serum of both the treatment groups due to subchronic exposure to thiacloprid. Subacute and subchronic exposure to 50mg/kg and 100mg/kg body weight doses of thiacloprid did not cause any significant change in serum bilirubin concentration for the treated rats as compared to control rats. At the end of 90 days of exposure of test chemical, marginal decrease in total bilirubin was observed (Figure 1.6).

Table 1.8 summarizes the effect of thiacloprid on LPO activity and antioxidant enzyme activity in liver of rats after 28 days of administration as compared with control. The liver lipid peroxide levels were high in the case of treated animals. LPO activity in terms of MDA level was found significantly increased by 100mg/kg body weight administration of thiacloprid ($p \leq 0.05$). Similarly a higher level of liver lipid peroxide in low dose treatment group was observed but, difference was not found statistically significant. Liver catalase level was found to be depleted significantly in HTD group of rats as compared to control group of rats ($p \leq 0.01$). Non significant change was found by administration of 50mg/kg body weight thiacloprid for 28 days. Liver superoxide dismutase activity was not found to be altered significantly after administration of thiacloprid in both treatment groups. Glutathione peroxidase activity was found elevated in both the treatment groups as compared to that of control rats, but values were not statistically significant. Activity of glutathione-S-transferase in HTD group was observed to be higher, but not statistically significant as compared to control group of rats. GST activity in LTD treatment group was not much different compared to control group of animals. Decreased level of reduced glutathione was observed in both the treatment groups as compared to control group, but the decrease was significant ($p \leq 0.05$) only for the high dose group (Figure 1.8).

Table 1.9 represents the subchronic effect of thiacloprid on liver LPO and stress marker enzyme activity. A statistically higher activity of LPO was found in both the treatment groups of rats ($p \leq 0.05$) as compared to control rats. Liver catalase level was found to be decreased in thiacloprid exposed rats as compared to control rats and activity was significantly lower in high dose group ($p \leq 0.01$). The low level of liver SOD in treated animals was found significant ($p \leq 0.05$) in HTD group and non significant in LTD group compared to reference group of rats. Lowered activity of GPx was found to be statistically significant in the 100mg/kg body weight treatment group as compared to control. GST activity was also observed to be lower in treatment group of rats with difference statistically significant for the HTD group ($p \leq 0.05$). Non enzymatic antioxidant level was also found to be depleted after subchronic intubation of thiacloprid with significantly low values for both LTD ($p \leq 0.05$) and HTD ($p \leq 0.01$) groups as compared to control group (Figure 1.9).

Histopathological changes in liver

Microscopic examination of liver of control rats showed normal structure of the central vein, radially arranged hepatocytes around the central vein and blood sinusoids (Figure 1.10 and

1.11). Histopathological investigation of liver sections of the treated rats showed disturbed hepatic lobule with variable degrees of hepatic degeneration in many areas of liver. Vacuolar hepatocyte degeneration and loss of radiating hepatocyte arrangement were very apparent (Figure1.12 to 1.15). Centrilobular hepatic damage (zonal necrosis) and focal area necrosis were observed in treated liver (Figure1.16). Infiltration of inflammatory cell was also seen (Figure 1.17).

DISCUSSION

Thiacloprid is a novel neonicotinoid insecticide belonging to sub class of nicotinyl compounds. It is used as a systemic insecticide for soil and foliar applications to control a variety of insect pests (EPA, 2003). The current study was designed to assess the toxicity potential of this pesticide on mammals, which are the non-target organisms, using SD rat as the animal model. Results of this study point towards significant incidence of toxicity in SD rats due to subacute and subchronic exposure to thiacloprid. These included nose bleeding, laboured breathing, reduced motility, ptosis and tremors. It was found that exposure to high dose of thiacloprid resulted in higher and cumulative toxic effects than the lower dose indicating a dose dependent toxic response towards the pesticide. Kammon (2010) had reported similar observations in layer chicken on exposure to another variant of neonicotinoid imidacloprid, providing support to the results of the present study. A decrease in body weight gain was also observed in animals exposed to thiacloprid daily at a higher dose of 100 mg/kg/body weight during the 90 days study period. In contrast, no change in the body weight was observed during subacute treatment of thiacloprid. Similar findings have been mentioned in the study reports of EPA (2003) and JMPR (2010), wherein a decrease of body weight gain was reported in male rats given 60mg/kg/day and 120mg/kg/day dose of thiacloprid.

The amount of food intake was also measured every day during the period of study. Lower food consumption by rats in the treatment group was observed during the subchronic study and this can be correlated to the earlier observation of reduced body weight in the treated animals. Observations made by Goyal (2010) during a toxicity study of thiacloprid in the digestive tract of birds, indicate that thiacloprid act as an irritant to the intestinal membrane, and opined that this could reduce food consumption. A similar response of the thiacloprid in rat intestine cannot be ruled out and might have resulted in reduced food intake in animals subjected to subchronic treatment. Hence, in the current study, overall low motility, general weakness and

low food intake could be the reasons for observed weight loss in animals exposed to thiacloprid during the treatment period.

Liver weight of thiacloprid treated rats was found to be increased during subchronic study. Liver enlargement can occur as a result of changes in dietary composition or metabolic aberration Liver enlargement without the accompanying histopathological change or functional impairment is often interpreted as being a physiological adaptation to enhanced workload or metabolic demand in body (Chopra and Griffin, 1985). The enlargement of the liver in the present study was probably due to the functional hypertrophy of the smooth endoplasmic reticulum and increased drug metabolizing multi-enzyme complex, as suggested by Zimmerman (1999). The reduced food consumption and increased liver weight in high dose exposed rats might be due to the toxic potential of neonicotinoid. White patches and necrotic bodies were commonly observed as an anatomical change in liver of treated male rats. This prominent necrosis in liver might be due to the transport of some toxic metabolite from intestine to liver and the presence of definite necrosis indicates towards the capability of the toxic metabolite in causing cell death. Similar anatomical changes in liver have also been observed by Shalaby (2010) in albino rats exposed to thiamethoxam, by Omiama (2004) in Japanese quail received imidacloprid and also by Kammon (2010) in layer chicken subjected to neonicotinoid imidacloprid.

Activities of serum enzymes like AST, ALT and ALP represent the functional status of liver (Mohany *et al*., 2011). High serum levels of AST and ALT are usually indicative of liver damage in animals (Durak *et al*., 1996) and humans (Ray and Drummond, 1991). In this study, there was a significant increase in the activity of serum enzymes, namely, ALT and AST in rats given orally the thiacloprid at the high dose of 100mg/kg body weight during both subacute and subchronic exposure regime. The level of ALT was also significantly higher in low dose treated rats (50mg/kg body weight) during the subchronic study. Freedland and Kramer (1970) suggested that enzyme levels are sensitive indicators of tissue damage, since they are liberated from cells even when the magnitude of lesions is not sufficient for morphological detection. Increased enzyme activity seen in the current study probably could be due to prolonged exposure to thiacloprid as a mechanism employed for detoxification of this pesticide. Increased enzyme activity may possibly be based on mutation of genes for the synthesis of these enzymes (Bolognesi and Morasso, 2000). Balani *et al*. (2011) who studied toxicity in male white leghorn chicks treated with different concentrations of neonicotinoid

imidacloprid have also reported an elevated level of transaminase enzyme. Shakoori *et al.* (1994) reported that the increase in plasma ALT activity is mainly due to the leakage of this enzyme from the liver cytosol into the blood. Further, alkaline phosphatase activity was also observed to be elevated in both the treatment groups during both subacute and subchronic studies.

Exposure to neonicotinoid imidacloprid reportedly induces hepatocyte damage and increases ALP, AST and ALT activity in serum of animals (Bhardwaj *et al*., 2010; Aydin, 2011; Toor *et al*., 2012). The metabolites of thiacloprid produced during its biotransformation may also be responsible for hepatotoxicity and the increase in serum activities of liver enzymes. Liver-ALP is mobilized most rapidly into blood and its levels in serum may increase at early period of liver damage. High ALP serum level is usually indicative of cholestasis which may also result in progressive liver disease like biliary cirrhosis (Alvaro *et al*., 2000). In the present study also similar increase in serum levels of all parameters have been observed, indicative of liver damage due to exposure to neonicotinoid thiacloprid.

In the current investigation, GGT activity was also found elevated in rats at 90 days of exposure to thiacloprid. GGT activity is a more sensitive marker for cholestatic damage than ALP and is very specific to the liver (McClatchey, 2002). Another biochemical marker used to evaluate liver function is lactate dehydrogenase (LDH) activity which was found to be increased in serum of thiacloprid treated groups of rats in present study. Increased LDH activity possibly indicates towards liver damage and it may be attributed to a generalized increase in membrane permeability, as reported by Kaczor *et al*. (2005). Moreover, elevation of LDH activity also indicates towards cell lysis and death as well as towards the switching over of aerobic glyoclysis to anaerobic respiration. The increased serum LDH activity may be due to hepatocellular necrosis leading to leakage of the enzyme and enzyme inhibition in liver which is supported by decrease in soluble protein (Shakoori *et al*., 1994). LDH can be used as an indicator of the potential of toxic agents to cause cellular damage (Bagchi *et al*., 1995). Similar results of increased LDH activity have been reported by Zaahkook *et al*. (2009) in Japanese quail after 3 weeks of imidacloprid exposure. Except for this study, literature concerning GGT and LDH activity after exposure to thiacloprid and any other neonicotinoid insecticides are lacking.

The total serum protein content was found to be higher in the rats exposed to different concentrations of the thiacloprid than the control during the 28 days study, but the difference was not statistically significant. However, significantly higher concentration of serum protein was observed in the treated rats after 90 days of thiacloprid exposure. A similar result has been given in EPA fact sheet (2003), where protein level increased after 6 weeks of thiacloprid exposure. Elevation in total protein content may be due to the hepatic detoxification, which results in the inhibitory effect on the activities of enzymes involved in detoxification. However, researchers have also reported a reduction in rat plasma protein due to exposure to different concentrations of neonicotionid imidacloprid (Zaahkook *et al*., 2009; Aydin, 2011). Significantly decreased serum glucose level was observed for the treated rats during both the studies. This observation finds support from a similar finding available in NRA (2001) public release summary and EPA (2003). Other such comparable reports on effects of thiacloprid on glucose levels are not available. Hypothetically, the low glucose level in the treated rats can be correlated to the low food intake in these animals as compared to the control group or it may be possible that thiacloprid affects the process of glucose absorption. Subacute exposure to thiacloprid did not cause any significant alteration in serum total bilirubin in the current investigation. However, subchronic oral administration of test compound decreased total bilirubin concentration in serum of rats. NRA (2001) public summary by Australia reported a similar finding but other research data regarding effects of this test compound on bilirubin are not available. However, Bhardwaj *et al*. (2010) have made a similar observation regarding bilirubin during a study conducted using the neonicotinoid imidacloprid on female rats.

Once thiacloprid enters in the biological system, it is transformed into primary and secondary metabolites. These intermediary metabolites, responsible for the hepatotoxic effect of test compound, may bind to cellular macromolecules and react with free amino groups of proteins; hence, the macromolecules may lose their physiological functions (Teppema *et al.*, 2002) or stimulate hepatocytes to produce more toxic metabolites (Durak *et al.*, 1996). They may cause cellular damage by covalent binding to cellular components such as enzymes, nucleic acids, and proteins or by any another mechanisms. Damage of cellular components may play an important role in death of liver cells (Fee *et al.*, 1979; Teppema *et al.*, 2002) and increased oxidative stress. Consequently, serum levels of AST, ALT, and ALP enzymes may increase. Besides, increased levels of liver LDH may result due to superoxide anions and hydroxyl radicals which cause oxidative damage to the cell membrane (Yadav *et al.*, 1997). Elevated free radicals and depressed antioxidant defense may lead to cell disruption, oxidative damage

to cell membrane and hence, increase susceptibility to lipid peroxidation (Kapoor *et al*., 2009; Kapoor *et al*., 2010). Elevated level of MDA, a product of lipid peroxidation (LPO), suggests an increased production of free oxygen radicals in rats (Mansour and Mossa, 2009). In the present work, thiacloprid significantly induced LPO and decreased other vital antioxidant enzymes in liver at high dose during subacute study and at both the doses during subchronic study. Susceptibility of liver to this stress due to exposure of thiacloprid is a function of overall balance between degree of oxidative stress and antioxidant capacity (Khan *et al*., 2005). High activity of LPO in liver in our study suggested the production of oxidative metabolites or free radicals during hepatic metabolism and this may be due to the progressive nature of free radical chain reaction. Increased level of MDA in liver tissues in the present study can be ably supported with similar results reported by Bhardwaj *et al*. (2010), Kapoor *et al*. (2010) and Aydin (2011) from studies conducted in rats using imidacloprid.

Oxygen free radicals and hydroperoxide collectively termed as reactive oxygen species (ROS) are produced by univalent reduction of dioxygen to superoxide anion (O_2^-) which in turn is converted into H_2O_2 and O_2 through a reaction catalyzed by SOD (Rai and Sharma, 2007). Antioxidant enzymes (SOD and Catalase) constitute the first line of defense against deleterious effect of oxyradicals in cells by catalyzing dismutation of superoxide radical. The observed decrease in SOD activity in liver of thiacloprid intoxicated rats during 90 days of exposure may be due to the consumption of this enzyme during conversion of O_2 to H_2O_2. Similar decreased activity of SOD was also reported with imidacloprid pesticide in rats (Kapoor, 2010; Duzguner and Erdogan, 2012). Finally, increased H_2O_2 resulting from catalase inhibition reduces SOD activity (Yu, 1994) in tissue. The current results were in accordance to these reports.

Various studies have demonstrated decrease in GPx activity due to xenobiotics (Kapoor *et al*., 2009; Mansoor and Mossa, 2009; Kapoor *et al*., 2010). Similar findings have been observed for the high dose treatment group during the present subchronic investigation. GSH plays a key role in the modulation of pesticide induced oxidative damage in tissues. GSH depletion evidently intensifies LPO and is known to predispose cells to oxidative damage (Khan *et al*., 2005). A significant depletion of GSH in liver in animals of both treatment groups during subchronic study and high dose group during subacute exposure together with decrease in activity of GPx may induce oxidative damage in liver tissue of rats. In addition, GSH participates in detoxification of xenobiotics as substrate for enzyme Glutathione-S-transferase

(GST) which is found to be reduced by thiacloprid in present investigation, which may be due to direct utilization of GSH as an antioxidant in terminating free radical reaction. Similar reduction in GSH has been reported earlier in rats with imidacloprid exposed liver as target organ (Gendy *et al*., 2010; Kapoor *et al*; 2010)

The decreased activities of SOD, GPx, Catalase and GSH together with increased LPO may have led to free radical toxicity during subchronic exposure to thiacloprid. Thiacloprid toxicity may also induce histopathological alterations in liver.

The increase in activities of specific liver enzymes and LPO activity correlate well with the gross and histopathological changes in treated liver observed in the present investigation. Increase in enzyme activity in serum may be due to enzyme loss in liver tissue (Bhardwaj *et al*., 2010). Histopathogical findings also support the gross anatomical changes like white necrotic patches and necrotic cyst observed as an anatomical change at the time of sacrifice. Goyal *et al*. (2010) observed marked degeneration of hepatocytes in thiacloprid exposed *Gallus domesticus* and also observed changes such as vacuolation and focal necrosis of hepatocytes. Similar changes have also been observed during the current study. The present information gathered on liver changes induced by thiacloprid is both limited and comparable with another neonicotinoid the imidacloprid (Bhardwaj *et al*., 2010; Kammon *et al*., 2010; *Toor et al*., 2012). Leucocytic infiltration was also observed by Toor *et al*. (2012) in female rats exposed to imidacloprid. Cyano group containing neonicotinoid thiamethoxam is also known to induce hepatic degeneration in liver tissue of albino rats (Shalaby *et al*., 2010).

CONCLUSION

The result of present study revealed that thiacloprid at low dose induces only subtle hepatotoxicity in rat when exposed for a short duration. However, subchronic exposure to thiacloprid significantly induced hepatic damage as evident from the increased levels ALT, AST, LDH and ALP enzyme activity and by generating oxidative stress. This may be due to disturbed cellular oxidative status as evidenced by increased LPO activity, decreased activities of SOD, catalase, GST, GPx and reduced GSH level in liver. Increased oxidative stress might have led to the histopathological changes observed in the study. Vacuolar hepatocyte degeneration and centrilobular necrosis were common histopathological changes observed in liver tissue which supports our presently observed deranged biochemical profile in the liver of thiacloprid exposed rats. Moreover, decreased food intake and increased liver weight in the

treatment groups may also be the result of thiacloprid intoxication. Thus, based on the results of the current study, it can be concluded that long term exposure to thiacloprid can potentially induce moderate to severe hepatic damage in the mammalian system.

Table 1.1. Weekly food consumption by rats per day during 28 days oral thiacloprid exposure

Group	Day 0 gm/day	Day 7 gm/day	Day 14 gm/day	Day 21 gm/day	Day 28 gm/day
Control	11.3±0.96[@]	11.7±1.2	11.6±1.56	12.6±1.6	12.8±1.47
Low Dose	10.9±1.5	10.6±1.8	11.42±0.9	11.7±1.59	12.43±1.24
High Dose	11.58±1.1	11.16±1.6	11.14±1.22	11.69±1.14	11.81±1.82

[@]Values are expressed as Mean ± SE; n=5 for each group

Table 1.2. Monthly food consumption by rats during subchronic thiacloprid oral exposure

Group	One month gm/day	Second month gm/day	Third month gm/day
Control	12±1.6[@]	14.95±0.98	16.53±1.44
Low dose	11.65±1.23	12.34±1.5	10.32±1.56↓*
High dose	11.46±1.42	10.42±1.87↓*	7.85±1.39↓**

[@]Values are expressed as Mean ± SE; n=5 for each group; * $p \leq 0.05$;** $p \leq 0.01$

Table 1.3. Effect of thiacloprid on animal weight and absolute liver weight after subacute and subchronic exposure

Group	Subacute		Subchronic	
	Animal weight (gm)	Organ weight(gm)	Animal weight(gm)	Organ weight(gm)
Control	280±8.9[@]	12.3±0.87	305±10.41	12.8±1.21
Low dose	278±8.99	12.19±1.2	276.9±11.6↓*	13.17±1.6
High dose	278.65±10.6	13.2±1.56	230.6±13.85↓**	13.58±1.57↑*

[@]Values are expressed as Mean ± SE; n=5 for each group; * $p \leq 0.05$;** $p \leq 0.01$

Table 1.4. Effect of thiacloprid on biochemical markers of liver function: Enzyme activities in the serum of control and treated SD rats after 28 days of exposure

Group	ALT IU/L	AST IU/L	ALP µM pNP release /mg tissue	GGT IU/L	LDH IU/L
Control	34.7±0.88[@]	10.9±0. 07	83.1±1.8	4.5±0.37	37.8±0.7
Low dose	36.4±0.87	11.8±0.51	94.5±2.3↑*	4.5±0.51	37.9±0.71
High dose	39.1±1.2↑*	12.5±0.63	99.3±3.3↑**	5.0±0.72	40.9±0.77↑*

[@]Values are expressed as Mean ± SE; n=5 for each group; * $p \leq 0.05$;** $p \leq 0.01$

Table 1.5. Effect of thiacloprid on biochemical markers of liver function: Enzyme activities in the serum of control and treated SD rats after 90 days of exposure

Group	ALT IU/L	AST IU/L	ALP µM pNP release /mg tissue	GGT IU/L	LDH IU/L
Control	37.2±1.13[@]	10.1±0.36	87.5±1.8	6.06±0.29	37.5±0.58
Low dose	42.1±1.16↑*	12.4±0.98	96.8±1.7↑*	7.99±0.35↑*	41.1±0.57↑*
High dose	46.7±1.1↑***[a]	13.8±0.62↑*	105.7±2.4↑***[a]	7.98±0.49↑*	42.4±0.82↑**

[@]Values are expressed as Mean±SE; n=5 for each group; * $p \leq 0.05$;** $p \leq 0.01$; *** $p \leq 0.001$; [a] significantly higher than low dose ([a] $p \leq 0.05$)

Table 1.6. Effect of subacute thiacloprid intoxication on serum biochemical parameters in SD rats

Group	Protein gm/dl	Glucose mg/dl	Bilirubin mg/dl
Control	4.7±0.29[@]	132.7±3.4	1.45±0.099
Low dose	5.2±0.35	105.4±4.8↓**	1.47±0.10
High dose	5.6±0.33	98.73±5.1↓***	1.47±0.11

[@]Values are expressed as Mean ± SE; n=5 for each group; ** $p \leq 0.01$; *** $p \leq 0.001$

Table 1.7. Effect of subchronic thiacloprid intoxication on serum biochemical parameters in SD rats

Group	Protein gm/dl	Glucose mg/dl	Bilirubin mg/dl
Control	5.7±0.15[@]	119.8±2.7	1.55±0.06
Low dose	8.2±0.47↑*	87.7±4.98↓***	1.45±0.12
High dose	7.99±0.77↑*	84.5±4.88↓***	1.37±0.10

[@]Values are expressed as Mean ± SE; n=5 for each group; * $p \leq 0.05$; *** $p \leq 0.001$

Table 1.8. Effect of thiacloprid on the activities of LPO, antioxidant enzyme and non enzymatic antioxidant after sub acute oral exposure in rat liver

Group	LPO (nmol/min/mg tissue)	Catalase µmole H_2O_2 liberate/ minute/ mg protein	SOD (% inhibition /min/mg tissue)	GST µmoles of GSH / minute/ mg protein	GPx (mM of GSH consumed/ mg tissue	GSH (µg/ gm tissue)
Control	17.9±0.77[@]	63.1±2.3	11±0.32	4.7±0.43	10.6±0.51	10.7±0.13
Low dose	21.5±1.06	66.3±2.2	10±0.43	4.6±0.59	12.8±0.98	9.5±0.35
High dose	22.1±1.22↑*	79.3±2.8↑**	11.6±0.47	6.3±0.63	12.7±1.1	8.7±0.41↓*

[@]Values are expressed as Mean ± SE; n=5 for each group; * $p \leq 0.05$; ** $p \leq 0.01$

Table 1.9. Effect of thiacloprid on the activities of LPO, antioxidant enzyme and non enzymatic antioxidant after subchronic oral exposure in rat liver

Group	LPO (nmol/min/mg tissue)	Catalase µmole H_2O_2 liberate/ minute/ mg protein	SOD (% inhibition /min/mg tissue)	GST µmoles of GSH / minute/ mg protein	GPx (mM of GSH consumed/ mg tissue	GSH (µg/ gm tissue)
Control	26.3±0.7[@]	27.9±0.8	14.7±0.54	5.4±0.28	12.6±0.37	10.2±0.36
Low dose	30.8±0.9↑*	25.5±0.9	13.7±0.45	4.5±0.57	10.9±0.54	7.98±0.54↓*
High dose	31.8±1.6↑*	22.5±1.1↓**	12.3±0.42↓*	3.5±0.31↓*	10.8±0.46↓*	7.29±0.39↓**

[@]Values are expressed as Mean ± SE; n=5 for each group; * $p \leq 0.05$; ** $p \leq 0.001$

Figure 1.1. Weekly food intake of SD rats during the study period of subacute exposure to thiacloprid

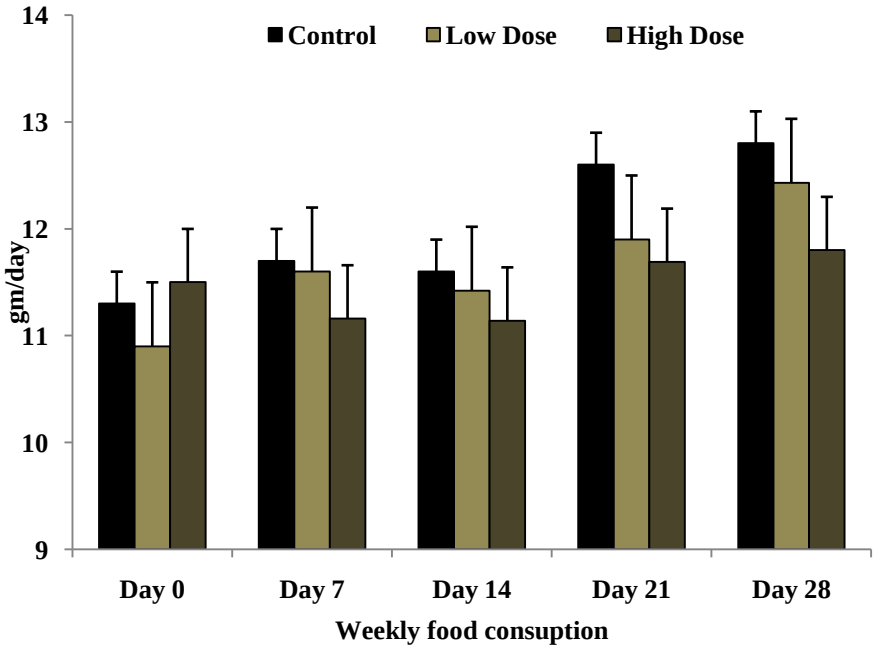


Figure 1.2. Monthly food consumption per day of thiacloprid exposed rat for 90 days

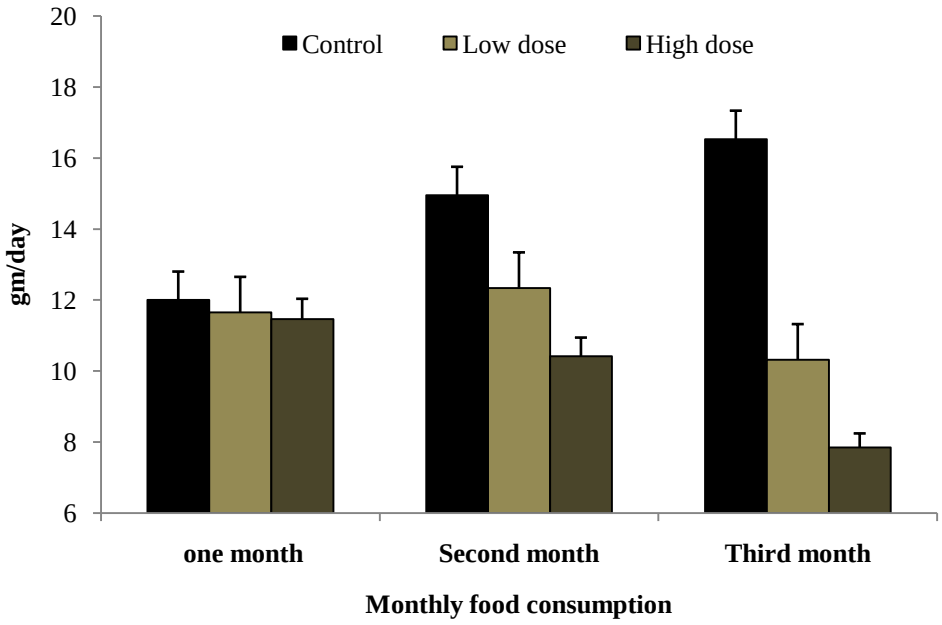


Figure 1.3. Animal weight and relative liver weight after 28 and 90 days of oral administration of thiacloprid

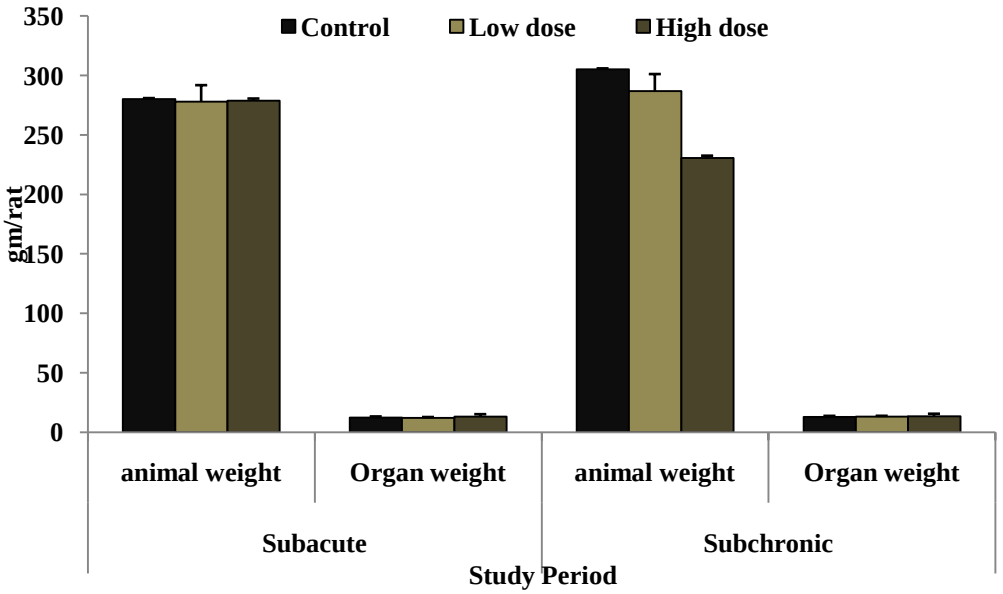


Figure 1.4. Serum enzyme parameters of rats after 28 days of thiacloprid administration

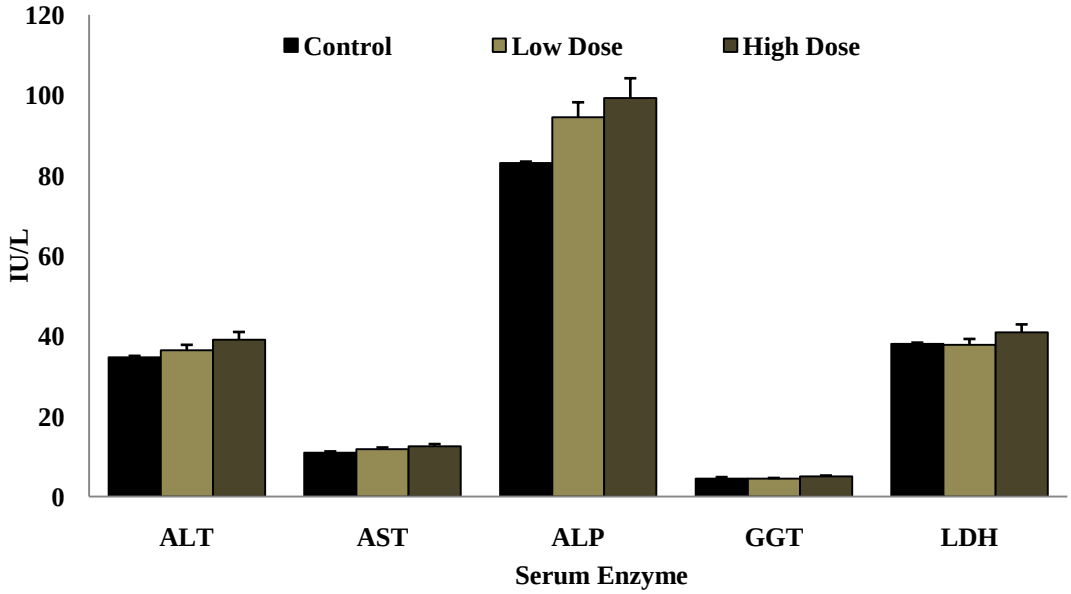


Figure 1.5. Serum enzyme parameters of rats after 90 days of thiacloprid administration

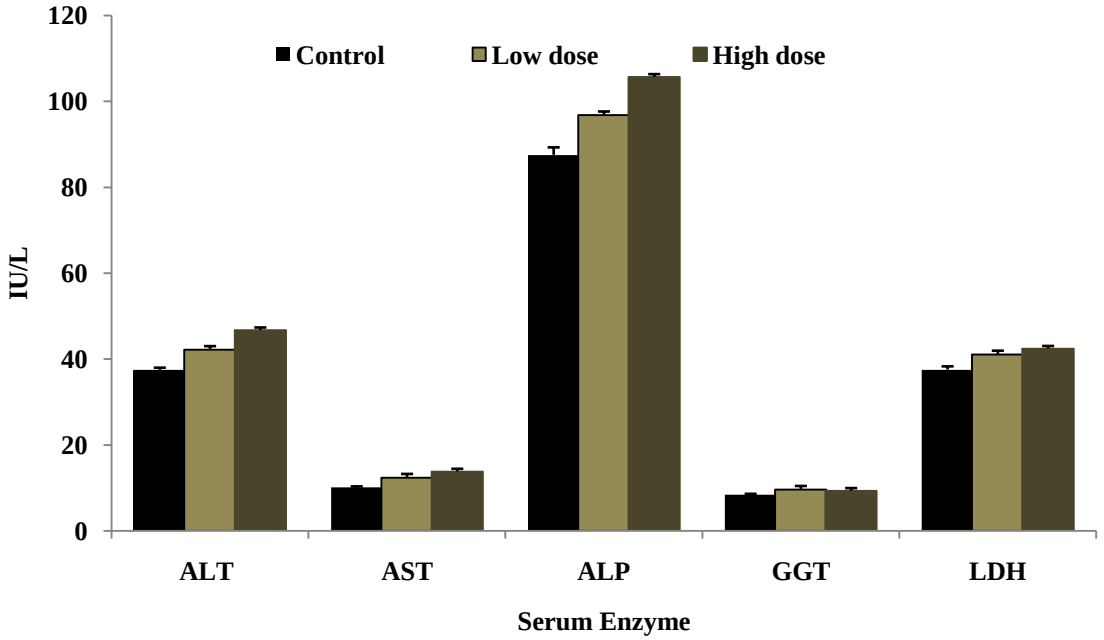


Figure 1.6 Efect of thiacloprid on Protein and bilirubin level in serum after subchronic and subacute exposure

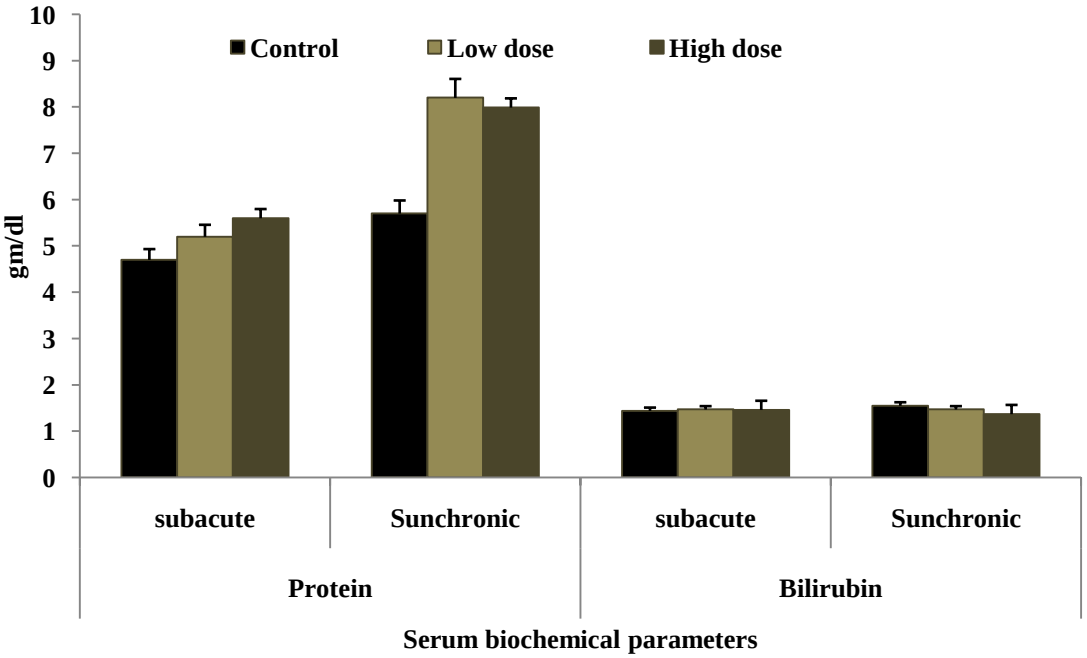


Figure 1.7 Serum glucose concentration after 28 and 90 days of thiacloprid oral exposure

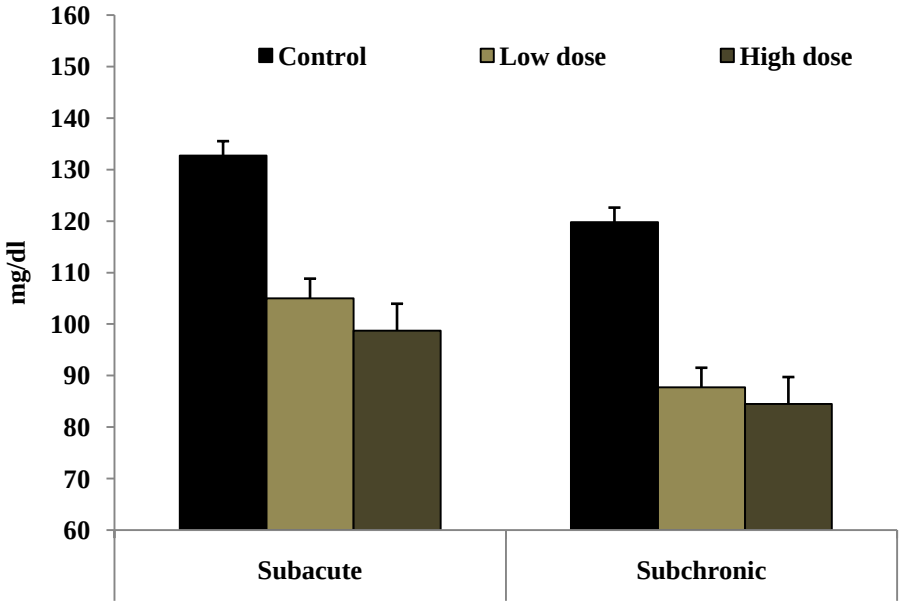


Figure 1.8. Activity of stress marker enzymes in thiacloprid exposed rat liver after 28 days of oral intubation

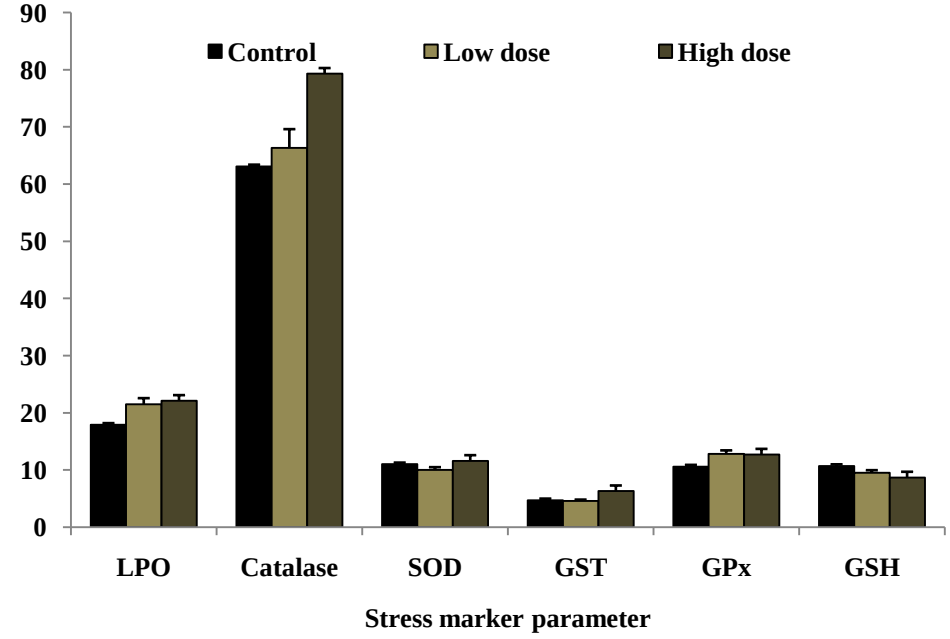


Figure 1.9. Activity of stress marker enzyme in thiacloprid exposed rat liver after 90 days of oral intubation

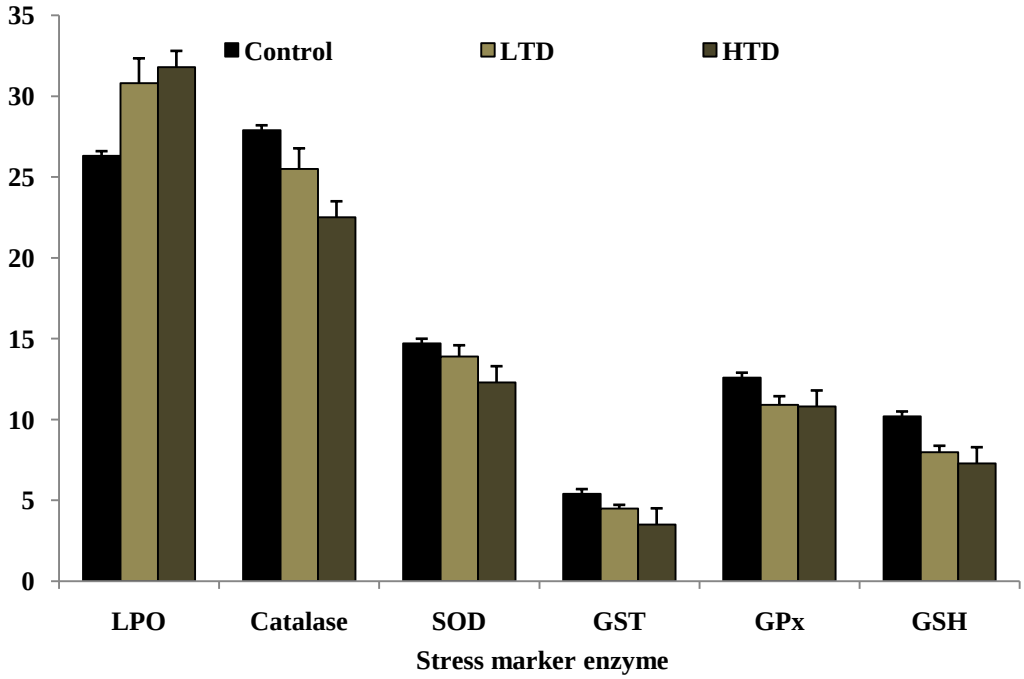


Figure 1.10 Liver tissue of control animals showing radially arranged hepatic cords around the central vein (CV) (H&E Stain, 10X).

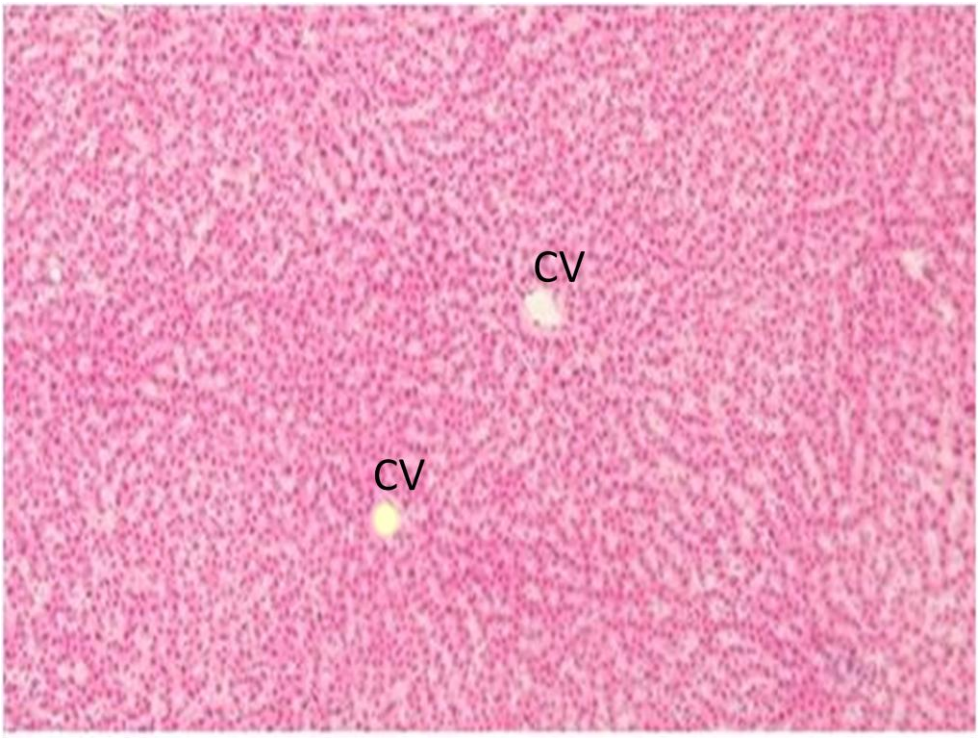


Figure 1.11 Liver tissue of control animals showing normal hepatocytes (H&E Stain, 100X).

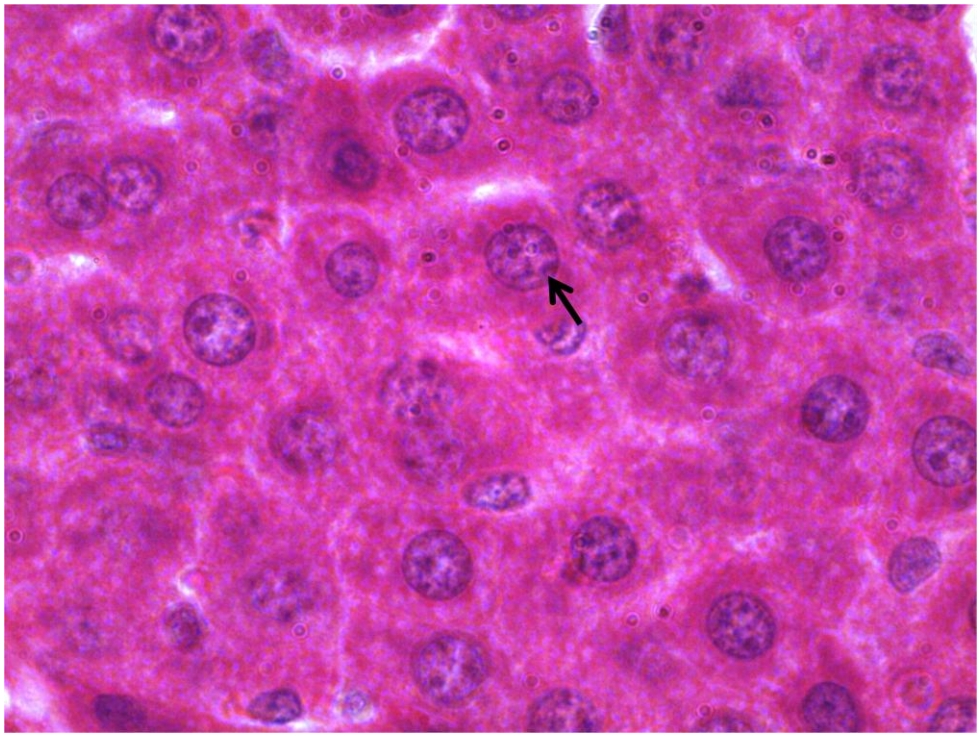


Figure 1.12 Thiacloprid treated rat liver section showing vacuolar degeneration with signs of necrosis (CV – central vein) (H&E Stain, 10X).

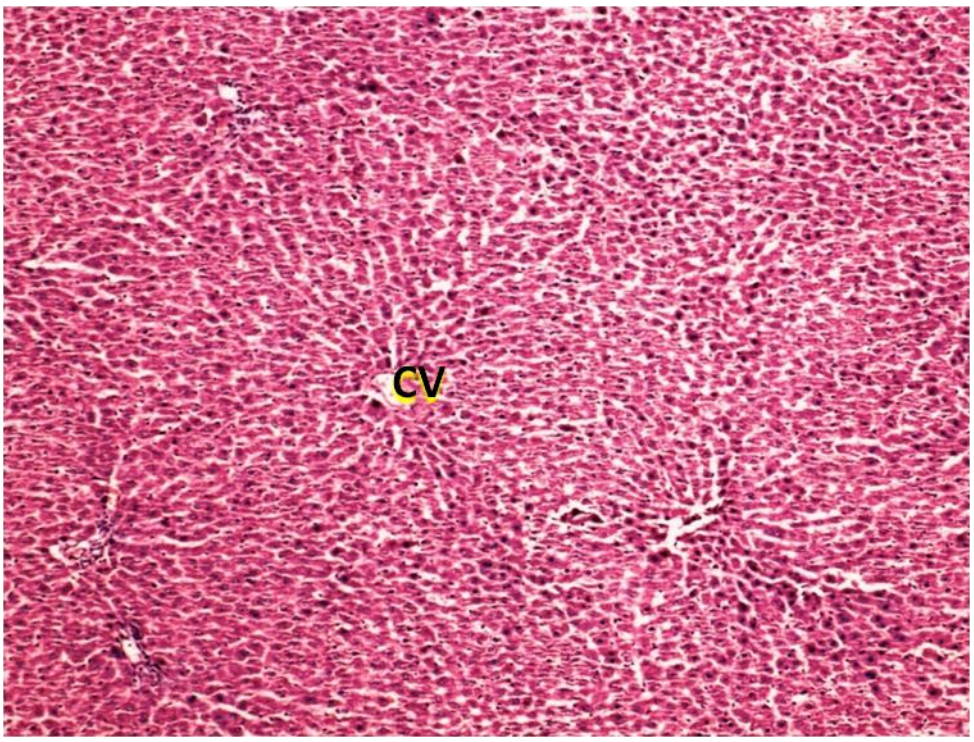


Figure 1.13 Thiacloprid intoxicated liver tissue showing vacuolar hepatocytes degeneration (arrows indicate vacuoles in hepatocytes (H&E stain, 100X).

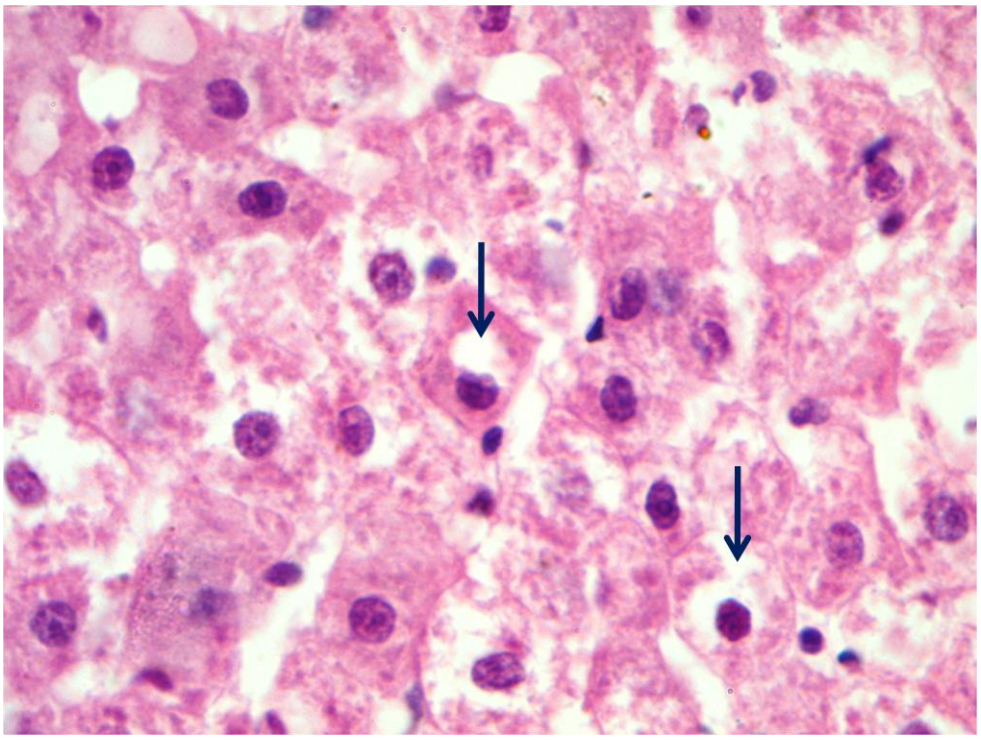


Figure 1.14 Histopathological appearance of thiacloprid treated liver tissue. Inset photograph with white necrotic spot on liver reflecting a necrotic patch in liver section (H&E stain, 40X).

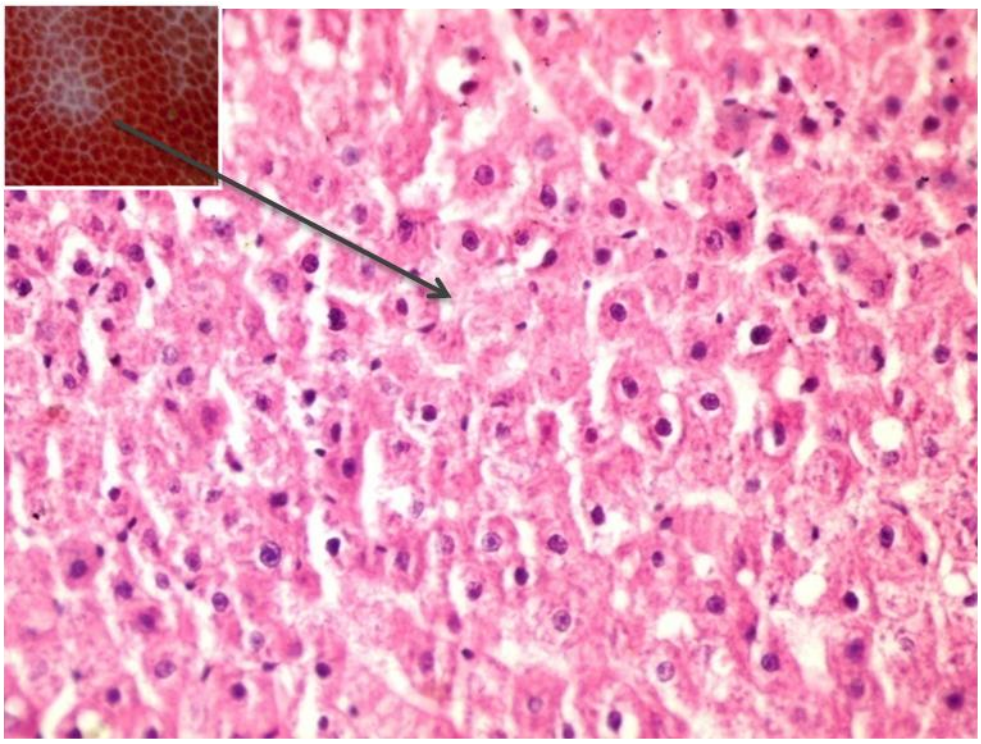


Figure 1.15 Histopathological alterations after thiacloprid intoxication. Inset photograph showing lesion of necrotic cyst seen as gross necrosis of hepatocyte (H&E stain, 100X).

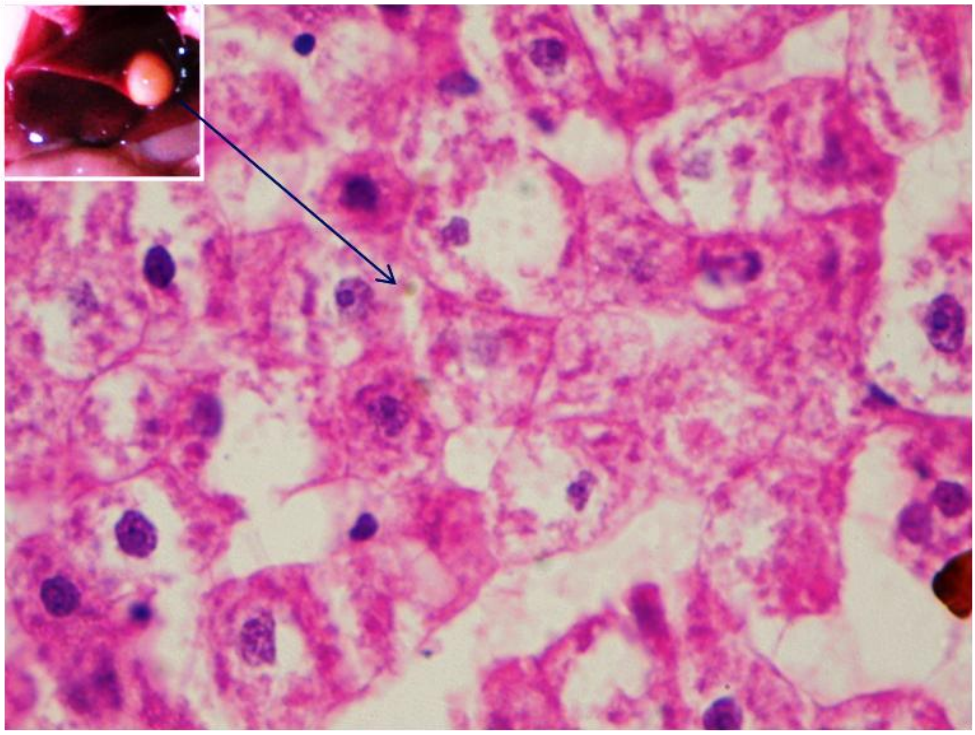


Figure 1.16 Thiacloprid treated rat liver showing centrilobular necrosis of hepatocyte surrounding central vein (black arrow) and central vein showing cellular debris of necrotic cell (H&E stain, 40X).

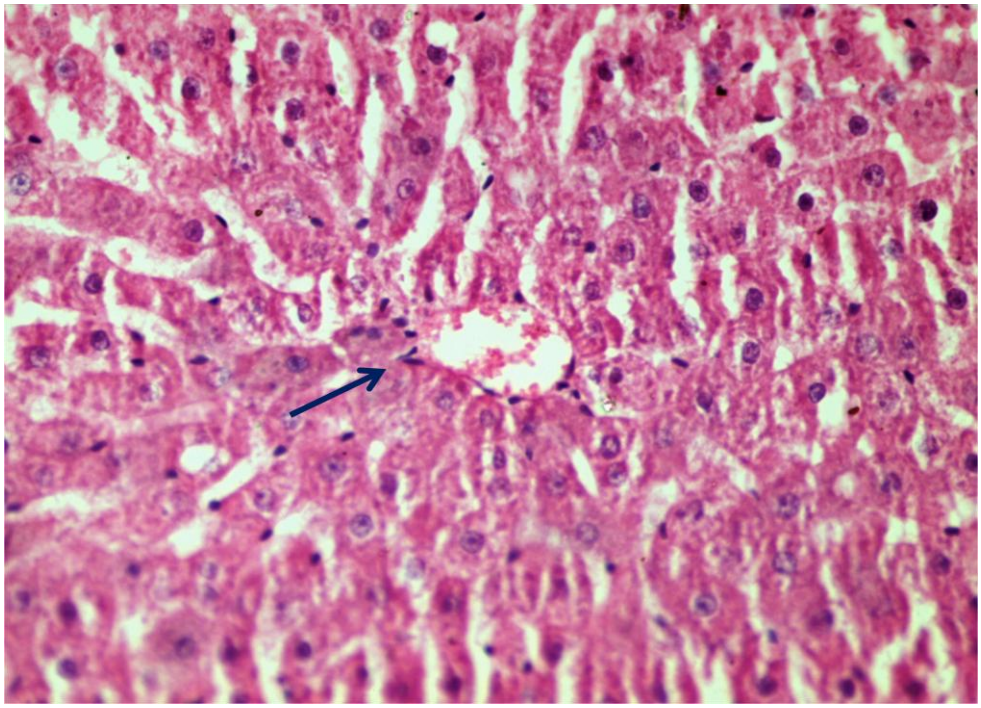
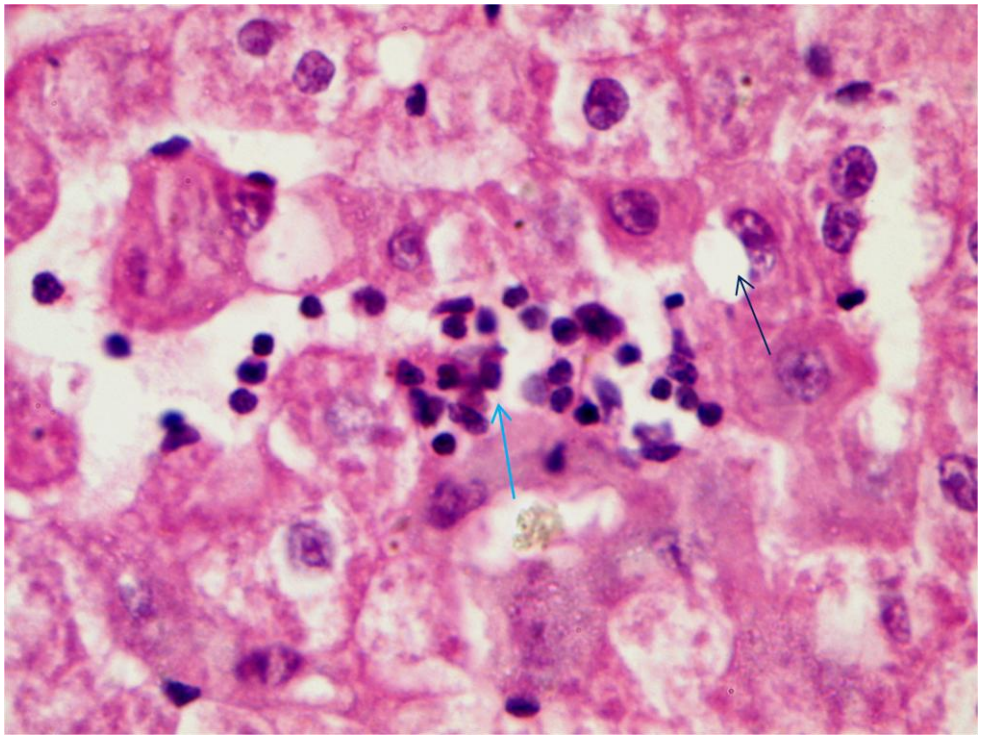


Figure 1.17 Thiacloprid treated rat liver showing infiltration of inflammatory cells (blue arrow), degenerating hepatocytes with vacuole (black arrow) and gross necrosis (H&E stain, 100X).



CHAPTER 2

THIACLOPRID INDUCES THYROID DYSFUNCTION IN RATS: EVIDENCES FORM REPEATED DOSE STUDIES IN MALE SPRAGUE DAWLEY RATS

INTRODUCTION

The mammalian thyroid gland is located on each side of the trachea. It consists of two elongated oval lobes, joined by a thin isthmus crossing the trachea. It is composed of two distinct endocrine cell populations. The C-cells (or parafollicular cells) are concerned with the production of calcitonin that regulates calcium metabolism, while the follicular cells produce and secrete the thyroid hormones (Noorden *et al*., 1977). Microscopically the gland is made up of thyroid follicles, surrounded by connective tissue. The follicles are composed of a single layer of cuboidal cells surrounding a lumen filled with colloid (NCM, 2002; Zoeller *et al*., 2007, Marta, 2011). These follicles synthesize the two major thyroid hormones; 3,5,3'-triiodothyronine (T_3) and thyroxine (T_4) (Zoeller *et al*., 2007).

In adults, the thyroid hormones (THs) stimulate metabolic rate in the liver, kidney, heart, nervous system and skeletal muscles (Randal *et al.*, 1997; Marta, 2011). T_3 is the more active of the two hormones, and the majority of biological actions of THs are believed to be mediated through receptors for T_3. In humans, the thyroid gland produces 100% of the T_4 found in the body, and 20% of the T_3. The other 80% is produced by peripheral conversion of T_4 to T_3 by deiodinase enzymes (Zoeller *et al*., 2007).

Thyroid hormones are lipid soluble and either bind to specific receptors on the inner mitochondrial membrane to activate energy metabolism, or to nuclear receptors to increase the transcription of specific genes, and ultimately alter the production of the proteins encoded by them (Marta, 2011). The thyroid hormones are cleared from the blood in the liver, following sulfonation by sulfotransferases (SULT) or glucuronidation by uridinediphosphate glucuronyltransferase (UDPGT). The modified THs are then eliminated through the bile (Zoeller *et al*., 2007). The synthesis of THs is regulated by the pituitary gland hormone thyrotropin also called as thyroid stimulating hormone (TSH). The levels of thyroid hormones

in the blood are regulated by a negative feedback mechanism involving the hypothalamic-pituitary-thyroid (HPT) axis (Zoeller *et al*., 2007).

A large number of xenobiotics are known to affect the thyroid hormone system known as thyroid disrupting chemicals (TDC). These include chemical classes like polychlorinated biphenyls (PCBs) and dioxins (PCDs), brominated flame retardants, ingredients in personal care products like UV-filters, hair dyes, antibacterial compounds and several pesticides (Brucker-Davis, 1998; Hurley *et al*., 1998; NCM, 2002; Crofton, 2008; Boas *et al*., 2009). The TDCs can perturb thyroid hormone homeostasis in a number of different ways. Some affect the thyroid gland directly, either by inhibiting the active transport of iodide into the follicular cell or by inhibiting the enzyme thyroid peroxidase, thereby affecting production of THs. Outside the thyroid, chemicals can cause transport disruption by altering binding to serum proteins.

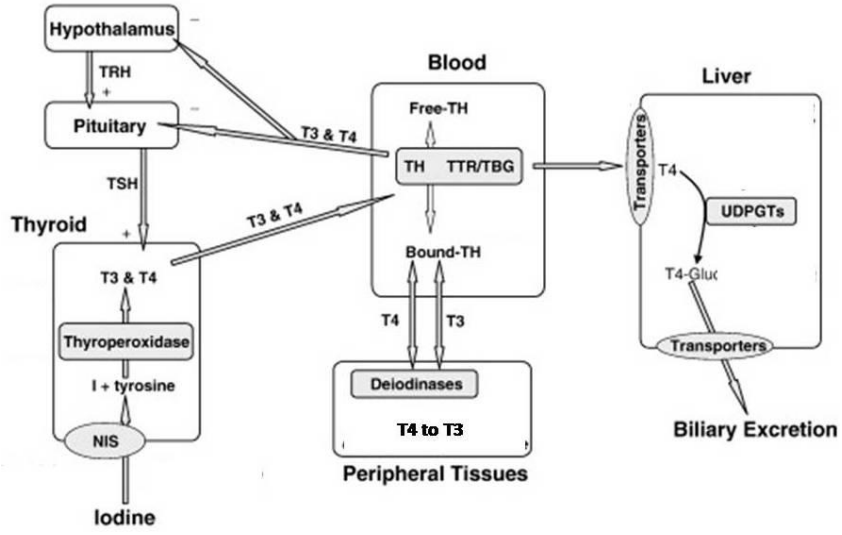


Figure A: Thyroid hormone control pathways and possible site for disruption (Crofton, 2008)

Other chemicals inhibit the conversion of T_4 to T_3 by affecting peripheral deiodinases, or enhance metabolism and biliary excretion of THs from the liver, through the action of the isoenzymes UDPGT or SULT (Hurley *et al*., 1998; Crofton, 2008). All these possible disruption mechanisms of thyroid hormone control are shown in Figure A.

In recent years, the hazards of using pesticides have been accentuated by the sharp rise in their use in agriculture and industry (Troudi *et al*., 2012). Neonicotinoid pesticides are currently among the most frequently used pesticides worldwide. NRA (2001) public summary suggested

that thiacloprid is a potent toxicant to thyroid gland. EPA (2003) recorded thyroid toxicity after oral and inhalation exposure of thiacloprid in both male and female rats.

After oral exposure of thiacloprid to rats, [^{14}C]methylene- or [^{14}C]thiazolidine-labelled thiacloprid was found to be rapidly and almost completely absorbed after a single high dose (100 mg/kg body weight), with maximum plasma concentrations of radioactivity occurring at 1–3 hours. Tissue residues at 48 hours after dosing were found in liver, kidneys, lung, adrenals and thyroid being the tissues with highest residues (PMRA, 1996; PMRA, 1997). Changes in circulating hormone concentrations (e.g. T_4, T_3 and thyroid-stimulating hormone, TSH) and effects on the rat thyroid (e.g. increased weight, hypertrophy and increased mitotic rate of follicular cells) were observed as a consequence of the liver enzyme induction in the study conducted by Bayer AgroSciences (PMRA, 2000). Thiacloprid administration developed thyroid toxicity via elevation of TSH and suppression of T_3 and T_4 synthesis which developed hypothyroidism in animals (EPA, 2003). Acute and subacute exposure of thiacloprid is also known to disturb the thyroid hormone level in serum of Wistar rats (Sekeroglu, 2012).

The thyroid hormones, triiodothyronine and tetraiodothyronine are necessary for appropriate energy levels and an active life of living organisms. It has long been known that thyroid hormones are of vital importance in maintaining the initial level of phospholipids in cell membranes and fatty acid composition of the lipids (Prasad and Kumar, 2005). T_3 plays a critical role in lipid metabolism by regulating genes involved in lipogenesis and lipolysis (Zhu and Chang, 2010). Hypothyroidism, characterized by low serum thyroid hormone levels, is associated with reduced metabolism, reduced lipolysis, weight gain, reduced cholesterol clearance, and elevated serum cholesterol. It is known that thyroid hormone has genomic and nongenomic effects (Davis *et al*., 2008). Overt hypothyroidism is associated with increased plasma cholesterol and triglyceride levels (Tulloch, 1974). Hypothyroidism may also exhibit elevated levels of high-density lipoprotein cholesterol (HDL-C), mainly due to increased concentration of cholesterol- and phospholipid-enriched HDL-2 particles (Pearce *et al*., 2008). Residues of thiacloprid are known to persist in thyroid gland of rat, causing thyroid gland hypertrophy by changing histoarchitecture and also increasing mitotic index of follicular cells (NRA, 2001). There is also evidence that hypothyroidism may directly affect the liver structure or function or vice versa (Van Steenbergen *et al*., 1989; Inkinen and Nordback, 2000). Recent studies have shown that the hepatic abnormalities associated with hypothyroidism can be

reversible over a matter of weeks with thyroxine replacement, with no residual liver damage (Huang and Liaw, 1995; Gaitan and Cooper, 1997).

From research done in animals so far, the relationship between thiacloprid induced thyroidal dysfunction and its physiological implications is still at large. A literature review has shown that no information is available concerning effect of thiacloprid on thyroid hormones. The only available information for the effect of thiacloprid on thyroid function is based on the data of the manufacturer (Bayer AgroSciences) and EPA factsheet in which evidence of oncogenicity (thyroid, ovary and uterus) in feeding studies in mice and rats has been demonstrated. Since data regarding influence on thyroid function from pesticide exposure are limited, toxicity assessment of thiacloprid in this regard deemed significant. Therefore, the specific purpose of the current study was to confirm whether the pesticide thiacloprid can disrupt thyroid function in a non-target mammalian animal system. Moreover, it would be interesting to know the effect of thyroid dysfunction on lipid metabolism and liver function since such a study can be of use in comprehending more meaningfully the general risks of exposure to thiacloprid.

To fulfill the above objective, the following parameters were assessed. 1) Evaluation of the structural and functional integrity of thyroid in SD rats after repeated thiacloprid intoxication. 2) Relevant markers were assessed to check whether altered level of thyroid hormone can interpolate lipid profile and disturb the lipid metabolism and 3) In order to understand the interplay, if any, between thyroid dysfunction and hepatic function and its implication on the behavioural pattern, efforts were made to compare the current results with that of chapter 1.

MATERIAL AND METHODS

The study was approved by the IAEC (Institutional Animal Ethical Committee) according to the norms of CPCSEA, India. A total of 30 healthy male SD rats (weight: 250–275gm; age: 7–8 weeks) were obtained from Sun Pharma Advanced Research Company (SPARC Ltd.), Baroda. Rats were housed in the departmental animal house (827/ac/04/CPCSEA) at controlled temperature (24 ± 2°C) and light-dark schedule (12:12) and were provided laboratory rat food (Pranav Agrochemicals, India) and water *ad libitum.* The animals were divided into three groups as control, low dose group (LTD) and high dose group (HTD), with 5 animals in each group. The commercial formulation of thiacloprid was diluted in distilled water to obtain desired dose concentrations. Rats were orally gavaged with 50mg/kg body

weight (LTD) and 100mg/kg body weight (HTD) thiacloprid every morning for duration of 28 days and 90 days. Any abnormal changes in behaviour were noticed and recorded.

The animals were sacrificed 24 hours after the last drug administration. Before sacrifice, animals were subjected to overnight fasting and blood was collected from the orbital sinus. They were sacrificed by cervical dislocation under mild diethyl ether anaesthesia. Thyroid gland was removed from the animals, washed in PBS, blotted free of fluids and weighed using a calibrated Sartorius analytical balance, following which it was fixed in 10% neutral buffered formalin for histological evaluation. Blood was kept for 2 hrs at 4ºC, and then centrifuged and serum was separated and was used for biochemical analysis.

Protocol I: Estimation of TSH and thyroid hormone

ELISA kit (GenWay Biotech, Inc.) of TSH, T_3 and T_4 was used for estimation of TSH, T_3 and T_4 level in rat serum. ELISA test of these hormones is based on the principle of a solid phase enzyme-linked immunosorbent assay. Mouse monoclonal anti-TSH, anti T_3 and anti T_4 antibody were used for solid phase immobilization (microtiter wells) and goat anti-TSH, anti T_3 or anti T_4 antibody was present in the antibody-enzyme (horseradish peroxidase) conjugate solution. The test sample was allowed to react simultaneously with the two antibodies, resulting in the TSH, T_3 and T_4 molecules being sandwiched between the solid phase and enzyme-linked antibodies. After incubation at room temperature, the solid phase was washed with water to remove unbound labelled antibodies. A solution of 3,3',5,5'-Tetramethylbenzidine (TMB) was added and incubated for 20 minutes, resulting in the development of a blue colour. The colour development was stopped with the addition of 1N HCl, and the resulting yellow colour was measured spectrophotometrically at 450nm using ELISA plate reader. The concentration of TSH, T_3 and T_4 is directly proportional to the colour intensity of the test sample.

Protocol II: Evaluation of lipid metabolism

1. **Lipid profile**

   Cholesterol concentration in serum was estimated by the method of Allain *et al*. (1974). The yield of coloured complex at the end of procedure was read at 505nm. The intensity of colour produced is directly proportional to the concentration of total cholesterol in the sample. The HDL-C and LDL-C was assayed using a Reckon diagnostic assay kit, based

on the method described by Grundy (1993) for measuring HDL and LDL cholesterol levels from serum or plasma. Absorption of HDL-C was measured at 690nm and LDL-C at 550nm.

2. Estimation of Triglyceride

Fossati and Lorenzo (1982) method was adopted for triglyceride estimation in serum. Intensity of purple coloured complex was measured at 546nm which is proportional to triglyceride concentration.

Protocol III: Histology of thyroid gland

Thyroid gland was removed and kept for overnight fixation in 10% neutral buffered formalin. Sample was dehydrated by keeping in series of increasing grade of alcohol, and then was cleared with xylene. Paraffin wax block was prepared and sections were taken using microtome. Sections were rehydrated in a reverse grade series of alcohol and stained with haematoxylin and eosin, followed by dehydration with alcohol. Sections were mounted in DPX and then evaluated for histoarchitectural changes under the microscope (Leica DM2500).

Statistical analysis

All data are expressed as mean ± standard error. The statistical significance of the mean differences between control and treated groups was analyzed by ANOVA, with Bonferroni post hoc test for multiple comparisons. Statistical calculations were performed with the SPSS for windows version 12.0. A probability value of ≤ 0.05 was taken as the cut-off value to consider differences as statistically significant.

RESULTS

The effects of thiacloprid on TSH, free T_3 (fT_3) and free T_3 (fT_4) hormones following 28 days of oral exposure are summarized in Table 2.1. TSH level was found to be marginally increased in both the treatment groups of rats as compared to the control group, but the difference was not statistically significant. However, a significant increase was observed for the high dose treatment group. No apparent change was observed in serum level of fT_4 hormone after 28 days of thiacloprid exposure. Nevertheless, significantly depleted level of fT_3 hormone was observed in the rats treated with 100mg/kg body weight of thiacloprid ($p \leq 0.05$) as compared to control animals. Value was not found significantly different for fT_3 hormone in LTD group as compared to reference animal group (Figure 2.1).

Table 2.2 summarizes the effect of commercial formulation of thiacloprid on the levels of TSH, fT_3 and fT_4 in the rat serum following 90 days of exposure. Subchronic oral treatment of thiacloprid increased the level of TSH in both the dosage groups of rats. The elevated values of TSH hormone in treated animals were found statistically significant for both LTD ($p \leq 0.05$) and HTD ($p \leq 0.01$) groups as compared to control value of TSH hormone. Also, significantly higher level of TSH hormone was noted in HTD group compared to LTD group of rats ($p \leq 0.05$). Level of fT4 in LTD group of rats was observed to be significantly lower than in control rats ($p \leq 0.05$). A significantly lower value was noted in HTD group of thiacloprid-exposed rats for fT4 hormone in serum, as compared to control ($p \leq 0.01$). Hormone level of fT3 was also found to be lower in LTD and HTD groups than the control animal group and the difference was observed to be significant for both high dose ($p \leq 0.01$) and low dose groups ($p \leq 0.05$) (Figure 2.2).

The data in table 2.3 summarizes values for lipid parameters in serum of male SD rats after 28 days of thiacloprid treatment. Cholesterol concentration was seen to be significantly ($p \leq 0.05$) higher in 100mg/kg body weight of thiacloprid exposed rats as compared to control. Concentration of cholesterol was also observed increased in low dose group but the difference was not significant. HDL-C level was found insignificantly increased in both the treated groups of animals compared to reference group. Significantly elevated level of LDL-C in serum was observed in HTD group of treated rats when compared to control rats ($p \leq 0.05$). LDL-C value of low dosage group was higher than the experimental control. Triglyceride concentration was noted to be higher in both the treatment groups of animals compared to the control animals, but value was only significant for higher dosage group ($p \leq 0.05$) (Figure 2.3).

The data represented in table 2.4 display the subchronic effect of thiacloprid on serum lipid parameters. Serum cholesterol level was significantly elevated ($p \leq 0.01$) for the HTD group than the control value after thiacloprid treatment for 90 days. In 50mg/kg thiacloprid treated rats, cholesterol was observed insignificantly higher than the experimental control. Both the dosage groups showed significantly higher concentration ($p \leq 0.05$) of HDL-C compared to the control concentration of HDL-C. Non significant higher value of LDL-C was observed for LTD group and significantly higher ($p \leq 0.05$) value was noted in HTD rats as compared to control animals. Subchronic exposure to thiacloprid led to significant increase in triglyceride concentration in both the groups of treated rats ($p \leq 0.05$) (Figure 2.4).

Table 2.5 shows the weight of thyroid gland in thiacloprid intoxicated groups during both the study periods. Weight of this endocrine gland was found to be increased in both the dosage groups of subchronic and subacute thiacloprid exposed rats as compared to control, but the increase was significant only for HTD group during subchronic study (Figure 2.5). Changes such as laboured breathing, ataxia, weakness, decreased body weight, nasal secretion and tremors observed during the earlier study (Chapter 1) were again recorded in the animals during both the study periods of the current investigation.

Histopathological changes in thyroid gland

Histological evaluation of control thyroid gland of rats revealed uniformly pink stained colloid with irregular pattern on the borders and acini were lined with single layer of cuboidal cells. The parafollicular cells were scattered in regular patches between thyroid follicles (Figure 2.6 & 2.7). Thyroid gland of treated rats was found to be enlarged due to thiacloprid intoxication. During histopathological evaluation, variable sized irregular follicles were seen lined with single layer of cuboidal cells partially or completely occluding the lumen (Figure 2.8). Haematoxylin and eosin staining showed that the follicles contained insufficient or no colloid. Some of the follicles were smaller than the normal (microfollicular pattern) and contained small amount of colloid while others were large enough and irregular (macrofollicular pattern) in shape. The colloid in these follicles stained more basophilic than normal. Follicular cell were found to vary from low cuboidal to flattened shape (Figure 2.9). Some active follicles were dispersed among hyperplastic follicles and parafolicular hyperplasia was also seen (Figure 2.10).

DISCUSSION

Environmental chemicals may interfere with thyroid gland function through different mechanisms of action, for example, at the receptor level, by binding to transport proteins, by cellular uptake mechanisms or by modifying the metabolism of thyroid hormones (Boas *et al*., 2006; Lacasana *et al*., 2010; Sekeroglu *et al*., 2012). Data on altered reproductive or endocrine function resulting from insecticide exposure are limited, but *in vivo* and *in vitro* studies show that some insecticides or their metabolites have been assessed for potential endocrine-disrupting activity and alteration of thyroid function (Akhtar *et al*., 1996; Tyler *et al*., 2000; Hu *et al*., 2002; Wang *et al*., 2002; Liu *et al*., 2006; Meeker *et al*., 2009). A number of pesticides are also thought to possess thyroid-disrupting properties in human and experimental animals (Garry, 2004; Toft *et al*., 2006; Meeker *et al*., 2009; Du *et al*., 2010; Lacasana *et al*., 2010;

Tebourbi *et al*., 2010; Villanger *et al*., 2011) and it has been reported that pesticide exposure in agriculture workers results in disturbances of thyroid hormone levels (Zaidi *et al*., 2000; Garry *et al*., 2003; Jeong *et al*., 2006; Toft *et al*., 2006; Lacasana *et al*., 2010; Sekeroglu *et al*., 2012).

There is a single study report by Sekeroglu (2012) about the effects of thiacloprid on thyroid hormones in experimental animals. Exposure to thiacloprid at 112mg/kg body weight and 22.5mg/kg body weight doses by gavage daily for 12 hrs and 4 weeks disturbed the function of thyroid gland and lipid metabolism. In the current study too, thiacloprid exposure for 28 days caused marginal increase in serum TSH and also decreased level of fT_3 hormone at the dose of 100mg/kg body weight. However, no such significant change in hormone profile was observed at the dose of 50mg/kg body weight. These results find support from similar findings reported by Sekeroglu (2012), who observed significantly increased TSH level and statistically non-significant decrease in the levels of fT_3 and fT_4 at 112mg/kg body weight of thiacloprid for acute exposure. Subchronic administration of thiacloprid also increased the serum TSH level and depleted serum fT_3 and fT_4 levels in present study indicating a state of hypothyroidism.

Recent evidence suggests that NO (Nitiric oxide) participates in the regulation of thyroid function. Hah *et al*. (2001) reported nitroarginine-containing dipeptide amides (Huang *et al*., 1999) and some peptidomimetic analogues (Huang *et al*., 2000) as potent and selective inhibitors of neuronal nitric oxide synthase (nNOS). The most potent nNOS inhibitor among these compounds is (4S)-N-(4-amino-5-[aminoethyl]aminopentyl)-N'-nitroguanidine. Nitroguanidine is one of the metabolites of neonicotinoids and it has ability to inhibit nitric oxide synthase. The diversity in biodegradable sites of neonicotinoids and multiple pathways insures against parent compound accumulation but provides intermediates reported to be active as nicotinic agonists and inducible nitric oxide synthase inhibitors (Ford and Casida, 2006). Nitric oxide is synthesised from its precursor L-arginine by the action of NOS (Malinski *et al*., 1993) which inhibits NO/cGMP pathway. Long-term inhibition of the NO/cGMP pathway affects parameters of thyroid hormone biosynthesis. A novel property of NO to inhibit thyroid peroxidase (TPO) and thyroglobulin (TG) mRNA expression is reported (Ruf and Carayon. 2006). The NO/cGMP action on iodine uptake could involve cGK (cGMP protein kinase) mediation which inhibits iodine uptake (Bazzara *et al*., 2007). Millatt *et al*. (1998) suggested a possible role for NO in the regulation of TPO activity and thus thyroid hormone synthesis. TPO inhibition cannot liberate iodine for addition onto tyrosine residue on thyroglobulin for the production of T_3 and T_4 and inorganic iodine is not oxidized into free iodine which

increases inorganic iodine concentration and leads to NIS (Sodium-Iodide symport) blockage. Therefore it is prudent to believe that the hypothyroidism observed in the thiacloprid treated animals could be a result of its metabolic intermediary nitroguanidine mediated hampered NO pathway.

Further, it is well documented that thyroid hormone plays an important role in the metabolism of lipids (Miyamoto *et al*., 1997). Hypothyroidism is usually associated with an increased serum concentration of total cholesterol and lipoproteins (Pucci *et al*., 2000). It is known that overt hypothyroidism is associated with increased fasting plasma cholesterol and triglyceride levels (Tulloch, 1974). Results of the current investigation also showed significantly increased serum cholesterol concentration after 28 and 90 days of thiacloprid treatment at the dose of 100mg/kg body weight. In 1952, Robertson and Kirkpatrick showed very high level of cholesterol in serum of patients with prolonged hypothyroidism due to reduced cholesterol clearance.

Serum HDL-C and LDL-C were significantly increased after thiacloprid administration for 90 days and levels of both were also comparatively higher in animals dosed at 100mg/kg body weight for 28 days compared to control group. The results of the present study find support from the NRA (2001) thiacloprid public summary. Nikkila and Kekki (1972) observed a moderate increase of serum triglycerides in hypothyroid condition (in humans), associated with a decrease in efficiency of triglyceride removal from plasma, which was attributed to a low lipoprotein lipase (LPL) activity. It is now widely recognized that hypothyroidism is one of the most common causes of secondary dyslipidemia. The most common abnormalities of lipoprotein metabolism associated with hypothyroidism are elevated levels of total cholesterol and low-density-lipoprotein cholesterol (LDL-C), which are attributable to the effect of thyroid hormone on lipoprotein lipase activity (Lithell *et al*., 1981) and the expression of the LDL-receptor (Staels *et al*., 1990). Hypothyroidism may also exhibit elevated levels of high-density lipoprotein cholesterol (HDL-C), mainly due to increased concentration of cholesterol- and phospholipid-enriched HDL-2 particles (Pearce *et al*., 2008). A decreased HDL-2 catabolism and cholesteryl ester transfer protein activity has been observed in hypothyroidism (Andrea *et al*., 2004). This decrease leads to a reduced transfer of cholesteryl esters from HDL to very-low-density lipoprotein (VLDL), thus increasing HDL-C levels (Dullaart *et al*., 1990). Similar reports regarding thiacloprid toxicity correlated with HDL and LDL levels in any animal model is still lacking.

Increased fasting plasma triglyceride levels commonly accompany hypothyroidism (Nikkila and Kekki, 1972; Tulloch *et al*., 1974). There was significantly higher triglyceride level observed in rats given subchronic treatment of thiacloprid in the current study and also in animals treated with a higher dose in the subacute study as compared to control animals. Nikkila and Kekki (1972) noted that in thyrotoxicosis the average plasma triglyceride level was slightly but significantly increased above that of control subjects. This change was associated with augmented production of triglycerides whereas the mean fractional removal rate was not different from normal. EPA (2003) toxicology data have also suggested the increased level of triglyceride after intoxication of thiacloprid.

The NRA (2001) public summary documented thyrotoxicosis caused by thiacloprid oral exposure to rats and exposure resulted in increased thyroid gland weight after 13 weeks of test compound administration. The results of thyroid weight recorded in the present study are similar to the findings of NRA public summary, whereby thiacloprid exposure to SD rats for 28 and 90 days was found to increase thyroid weight.

There is also evidence that hypothyroidism may directly affect the liver structure or function and similarly chronic liver damage may develop thyroid dysfunction. Hypothyroidism has been associated with reduced bilirubin and bile excretion. In experimental hypothyroidism, the activity of bilirubin UDP-glucuronyltransferase is decreased, resulting in a reduction in bilirubin excretion (Van Steenbergen *et al*., 1989). The reduction in bile flow may be in part due to an increase in membrane cholesterol-phospholipid ratio and diminished membrane fluidity (Van Steenbergen *et al*., 1989), which may affect a number of canalicular membrane transporters and enzymes, including the Na^+/K^+-ATPase and alkaline phosphatase. The triad of reduced bilirubin excretion, hypercholesterolemia and hypotonia of the gall bladder seen in hypothyroidism increases the incidence of gallstones (Inkinen and Nordback, 2000; Malik and Hodgson, 2002). As observed in our previous study, thiacloprid exposure decreased bilirubin level and increased ALP activity (Chapter 1) and this development of hepatotoxicity can be correlated to the altered thyroid gland function observed in the present study. Also, several hormones are known to affect liver size in the rat. Thyroid hormones are reported to elicit hypertrophic and hyperplastic responses in the liver (Huang and Liaw, 1995). This report prompts us to correlate the hypothyroidic condition observed in the present study and liver enlargement encountered in the previous study (Chapter 1)

Histological evaluation of the thyroid gland showed that rats given 3 months oral exposure to thiacloprid developed structural malformation in the thyroid gland. This was indicated by reduction of connective tissue in interfollicular space as well as by completely absent or scanty colloids in follicles. The number of follicular epithelial cells had increased, layers of which were extremely flattened in some areas of the gland. Histopathological changes of thiacloprid-intoxicated thyroid clearly suggested follicular epithelial hyperplasia and mild to moderate parafollicular hyperplasia. NRA public summary (2001) had reported a similar kind of histopathological description of rat thyroid gland after thiacloprid administration. Summary report also concluded hypertrophy of follicular epithelium and increased mitotic rate of follicular cells in thiacloprid exposed rats. FAO (2006) has also suggested the possibility of thyroid adenoma development due to thiacloprid intoxication.

Notwithstanding the above structural and functional impairment to thyroid, during the present work, abnormal behavioural changes like tremors and ataxia were commonly noted in the thiacloprid treated animals. Aron (2005), Milanov and Sheinkova (2000) reported that tremor, muscle weakness and ataxia are well-known symptoms of thyrotoxicosis in humans. Public summary of NRA (2001) also suggested similar symptoms in rats after 3 weeks of thiacloprid exposure. Due to lack of supportive data regarding such changes in rodent or any other mammalian animals, results of the current study have not been discussed elaborately. However, comparable behaviour after thiacloprid exposure has been reported in honey bees which included changes such as shaking and tremors, uncontrolled and uncoordinated movements, staggering, inability to take up a correct position of the body, and prolonged frenetic movement of the legs and rotation when in the supine position (Daniela *et al*., 2011; Vidau *et al.*, 2011). Observations of changes such as tremor and ataxia are important as they may develop due to neuronal impairment also. So these symptoms can be correlated with possible neurotoxic effects of thiacloprid on the mammalian system, which forms the mainstay of the next study.

CONCLUSION

Repetitive dosage of thiacloprid may disturb thyroid hormonogenesis by alteration in thyroid hormone level through possible inhibition of NO pathway by one of its intermediate metabolite in the mammalian system. This thyrotoxicosis condition may be responsible for elevation of TSH and depleted level of fT3 and fT4. Decreased level of thyroid hormone interpolated lipid metabolism and hence, cholesterol, HDL-C and LDL-C were found to be increased in serum of

thiacloprid treated rats. It has also been reported that hypothyroidism, characterized by low serum thyroid hormone levels, is associated with reduced metabolism, reduced lipolysis, reduced cholesterol clearance, and elevated serum cholesterol and triglyceride (Davis *et al*., 2008) which may induce pathophysiological change. Similar observations made in the current study explain the aetiology of histopathological changes observed in the thiacloprid-intoxicated thyroid gland. Disturbance in thyroid physiology can also be ably correlated to changes in liver function observed in the previous chapter. Therefore, it can be evidently concluded from the findings of present work that thiacloprid has the potency to develop thyrotoxicosis directly by down regulating various steps of thyroid biosynthesis which are under the regulating of a potentially hampered NO signalling hence, promoting hypothyroidism in rat. The later condition in turn downplay the lipid metabolism and hepatic function resulting in further structural anomalies culminating in developing abnormal behavioural response in the thiocloprid intoxicated animals.

Table 2.1. Effect of subacute thiacloprid administration on serum TSH and thyroid hormone in rats

Group	TSH µU/ml	fT_4 ng/ml	fT_3 mcg/dl
Control	0.53±0.01[@]	12.6±0.32	1.3±0.037
Low Dose	0.56±0.07	11.7±0.58	1.15±0.042
High Dose	0.72±0.08	11.5±0.47	1.1±0.052↓*

Table 2.2 Effect of subchronic thiacloprid treatment on serum TSH and thyroid hormone in rats

Group	TSH µU/ml	fT_4 ng/ml	fT_3 mcg/dl
Control	0.57±0.03[@]	12.2±0.47	1.27±0.05
Low Dose	2.20±0.18↑*	10.2±0.48↓*	0.96±0.07↓*
High Dose	3.52±0.53↑**[a]	9.5±0.57↓**	0.8±0.08↓**

Table2.3. Effect of thiacloprid on serum lipid parameters after 28 days of intubation

Group	Cholesterol mg/dl	HDL-C mg/dl	LDL-C mg/dl	Triglyceride mg/dl
Control	126.6±3.9[@]	49.8±1.2	42.0±1.3	30.5±0.56
Low Dose	135.1±5.8	55.5±2.4	48.9±2.1	32.5±0.62
High Dose	154.8±7.8↑*	59.0±3.4	50.5±2.5↑*	33.7±0.71↑*

Table 2.4 .Effect of thiacloprid on serum lipid parameters after 90 days of oral exposure

Group	Cholesterol mg/dl	HDL-C mg/dl	LDL-C mg/dl	Triglyceride mg/dl
Control	110.7±2.5[@]	44±2.7	45.7±2.2	44.5±0.96
Low Dose	125.4±4.4	58±3.1↑*	50.8±2.1	51.1±1.93↑*
High Dose	133.7±5.3↑**	61±4.5↑*	56.4±2.9↑*	53.1±1.94↑*

Table 1.5. Percent relative weight of thyroid gland in thiacloprid intoxicated SD rats

Group	Control	Low dose	High dose
Subacute	3.6±0.02	3.7±0.06	3.8±0.05
Subchronic	3.4±0.02	3.6±0.07	3.6±0.08↓*

[@]Values are expressed as Mean±SE; n=5 for each group; * $p \leq 0.05$; ** $p \leq 0.01$; [a] significantly higher than low dose ([a] $p \leq 0.05$)

Figure 2.1. Effect of thiacloprid on TSH and thyroid hormone after 28 days of oral exposure

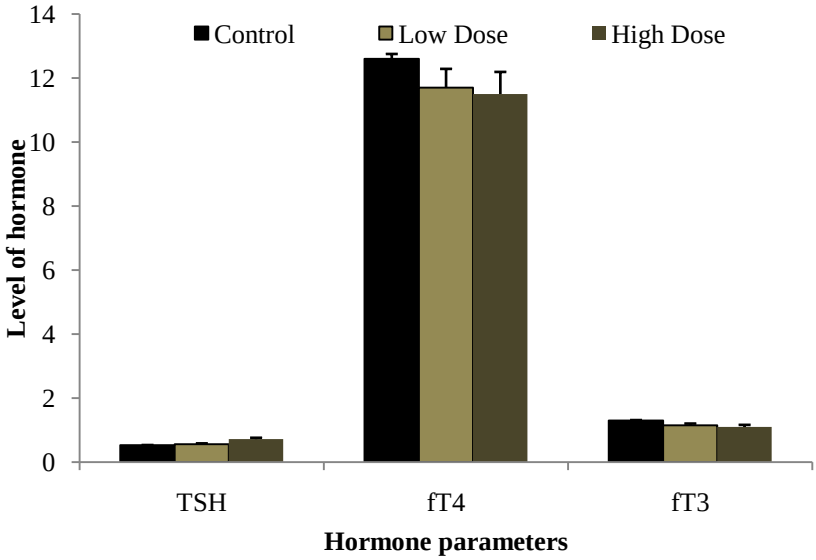


Figure 2.2. Subchronic effect of thiacloprid on serum TSH and thyroid hormone of SD rats

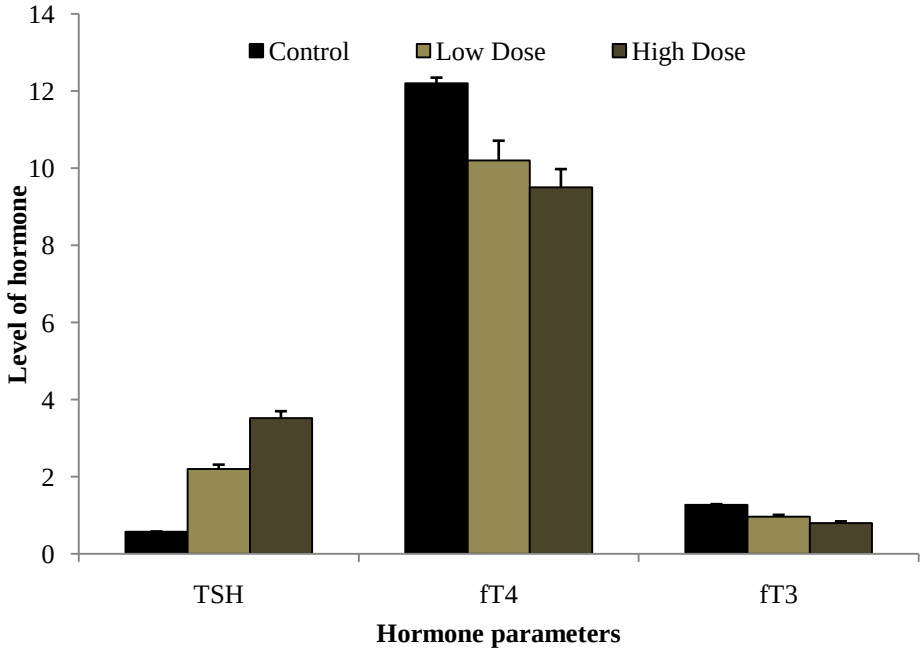


Figure 2.3. Effect of thiacloprid on serum lipid parameters after 28 days of intubation

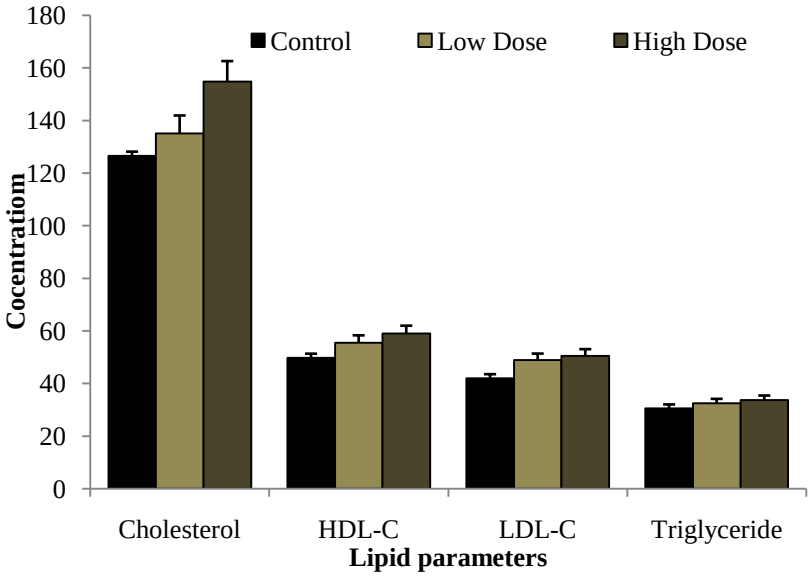


Figure 2.4. Effect of thiacloprid on serum lipid parameters after 90 days of oral exposure

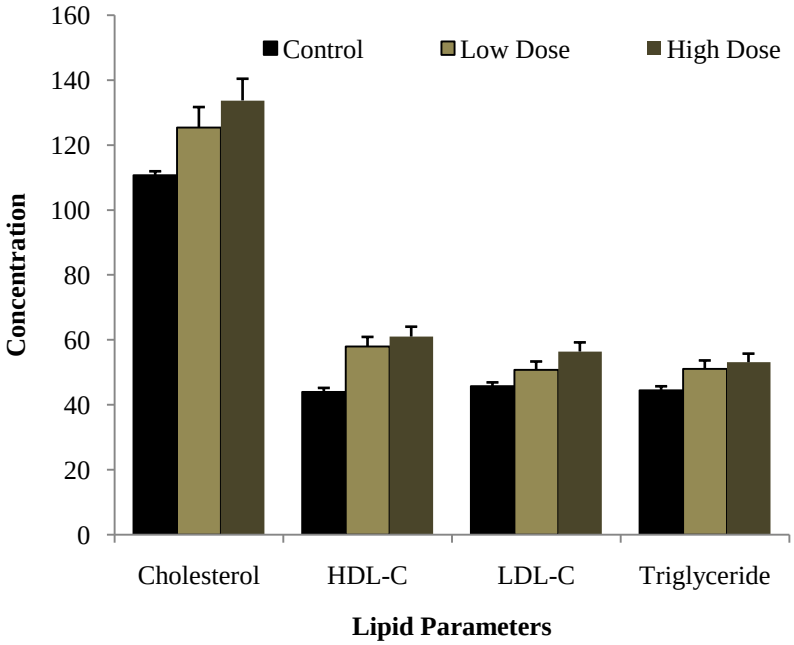
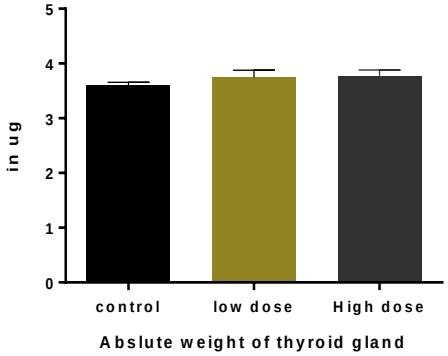


Figure 2.5 Effect of thiacloprid on serum lipid parameters after 90 days of oral exposure: Relative weight of thyroid gland after thiacloprid intoxication 1). Subacute changes 2) Subchronic changes

1)



2)

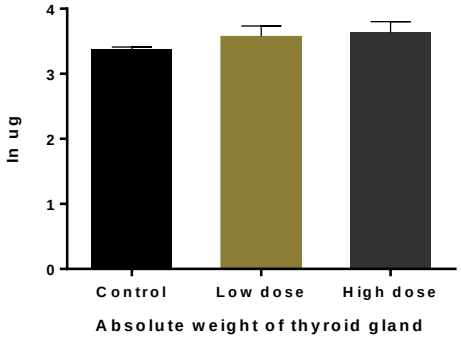


Figure 2.6 Thyroid gland of control SD rat. Arrow showing active thyroid follicle with colloid (H&E stain, 20X).

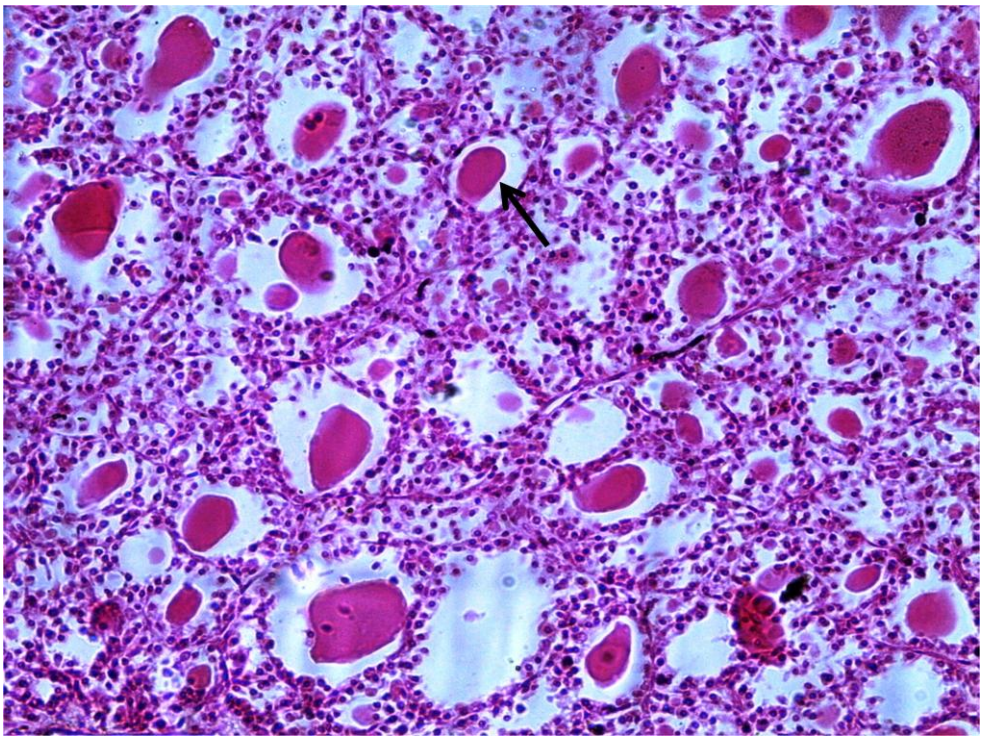


Figure 2.7 Active follicle of thyroid gland showing high cuboidal cell (black arrow) of control rat (H&E stain, 100X).

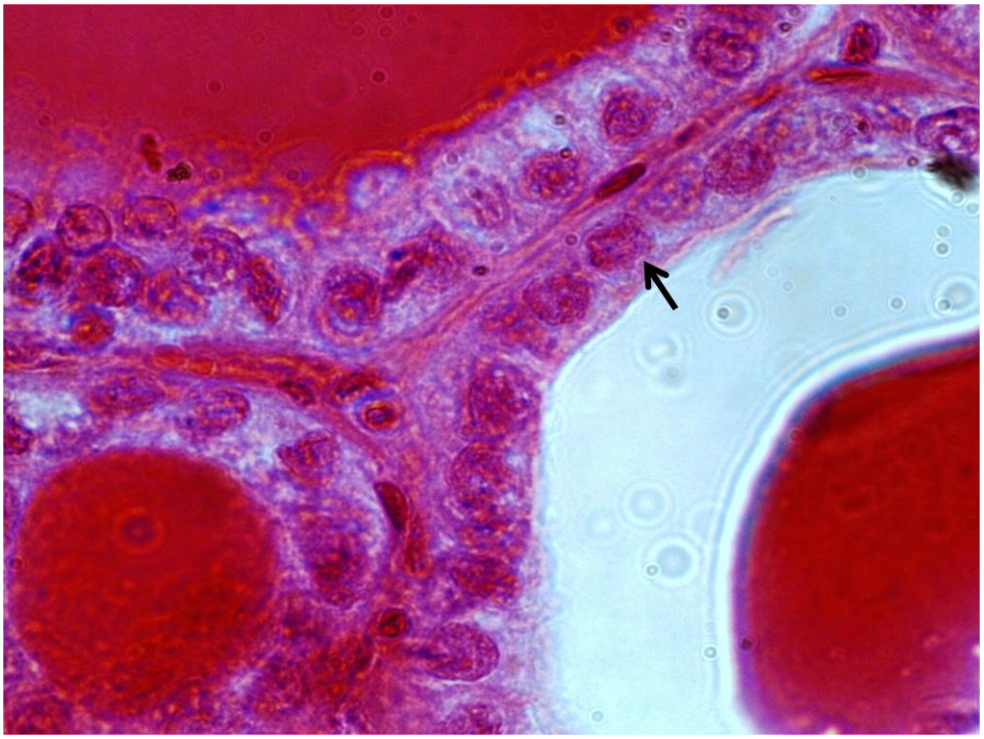


Figure 2.8 Thiacloprid treated thyroid gland of SD rats showing variable size of thyroid follicle (asterisk) with scanty colloid (blue arrow) or no colloid (green arrow) ((H&E stain, 20X).

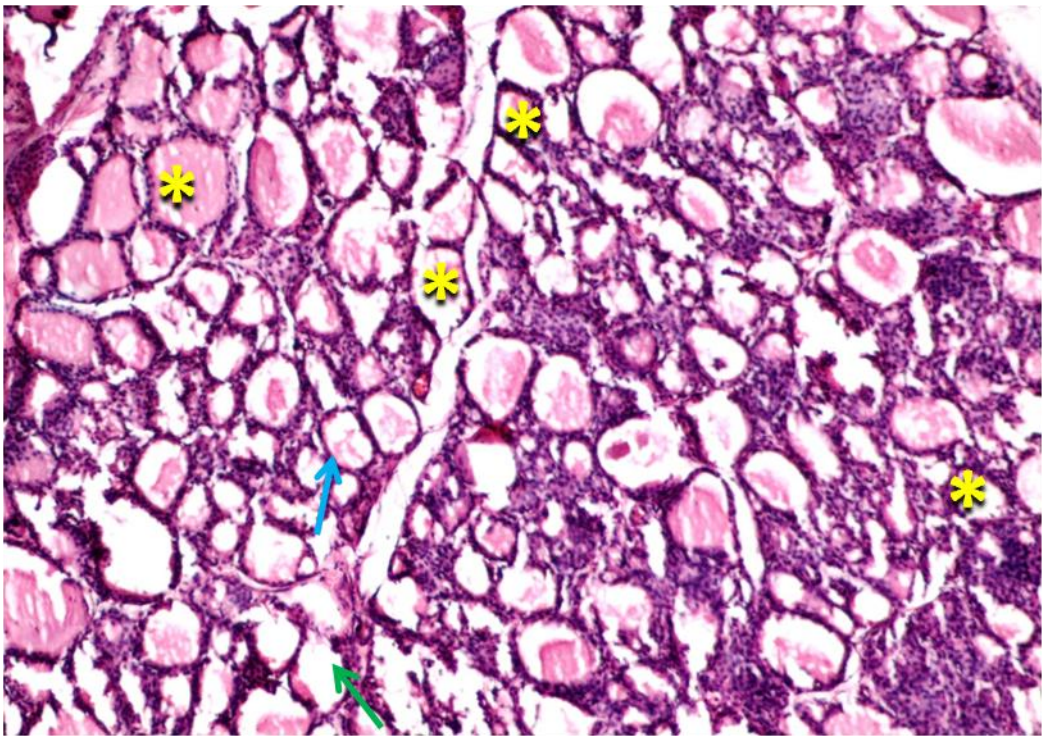


Figure 2.9 Thiacloprid treated rat throid showing hyperplasia with low cuboidal cells (green arrow) and flattened cuboidal cells of follicular epithelium (blue arrow) (H&E stain, 100X).

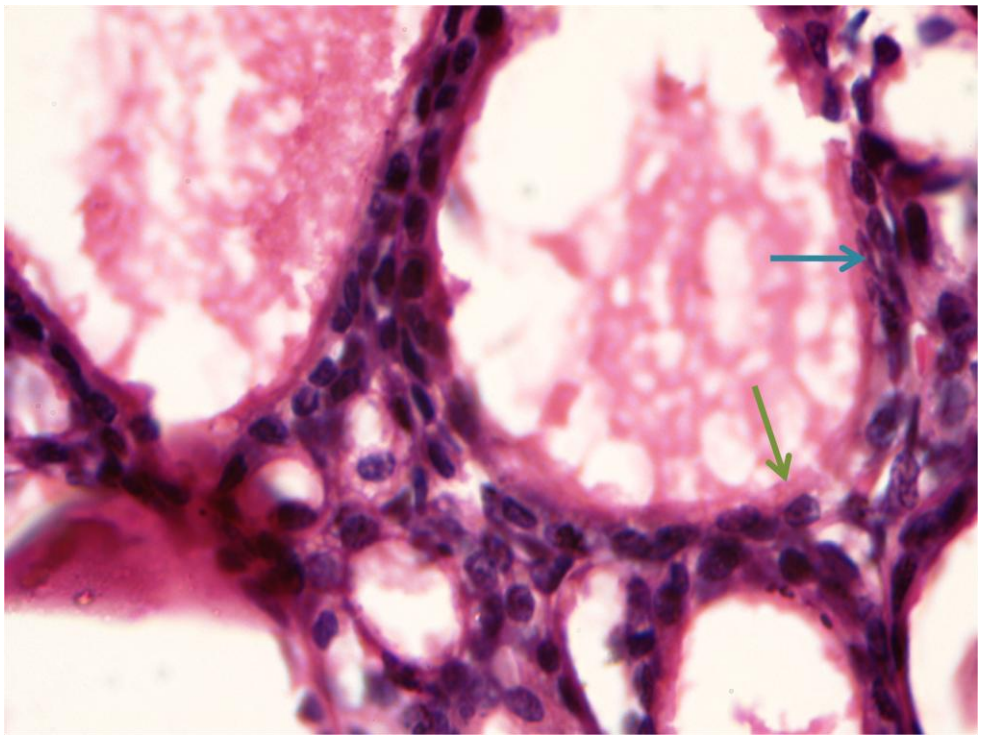
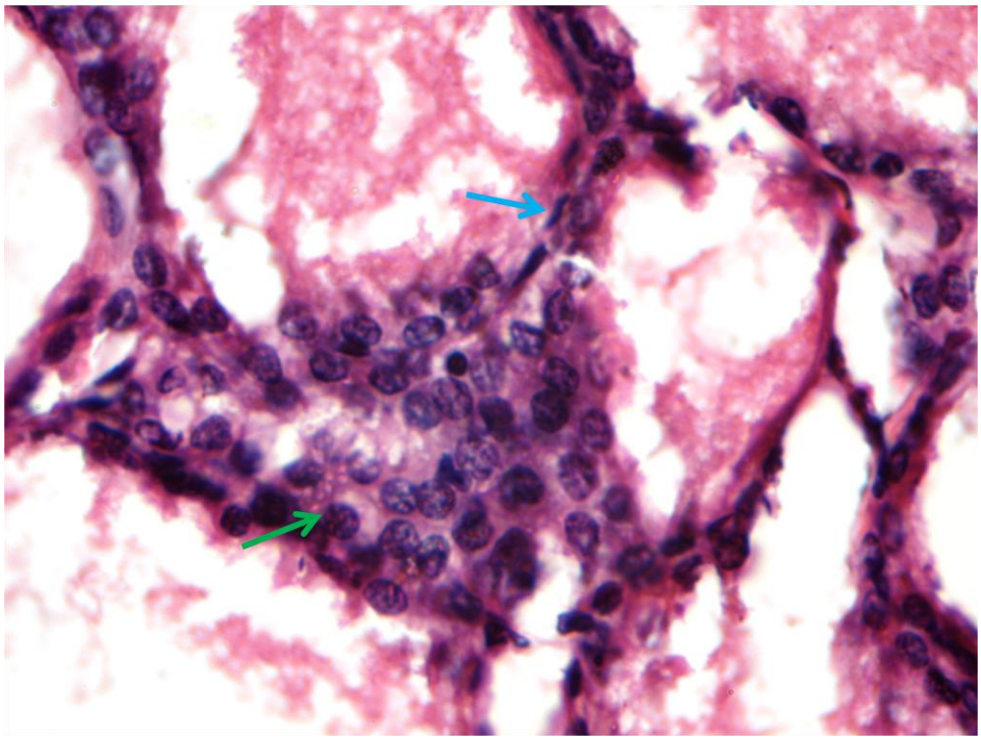


Figure 2.10 Thiacloprid treated rat thyroid showing parafollicular hyperplasia (green arrow) and flattened cuboidal follicular epithelial cells (H&E stain, 100X).



CHAPTER 3

EVALUATING THE THIACLOPRID INDUCED TOXICITY TO NERVOUS SYSTEM

INTRODUCTION

Neonicotinoids, the most important new class of synthetic insecticides of the past three decades are used in various agricultural fields worldwide (Tomizawa and Casida, 2003). Global annual trade in neonicotinoids is to the tune of one billion dollars that accounts for 11%–15% of the total insecticide market. They are readily absorbed by plants and act quickly by targeting the receptors of neurotransmitters, at low doses, on piercing-sucking insect pests (aphids, leafhoppers, and whiteflies) of major crops and companion animals (Tomizawa and Casida, 2005).

Ligand-gated ion channels (LGICs) are receptors with integral ion channel mediating the fast action of neurotransmitters at synapses in vertebrate and insect nervous systems. Prolonged activation, modulation, or inhibition of LGICs, irrespective of the receptor type involved, can result in toxicity. Neonicotionoids are a commercially important class of insecticides that target LGICs like nicotinic acetylcholine receptors (nAChRs) of insects in a selective manner (Matsuda *et al*., 2001). Neonicotinoids are synthetic, nicotine-derived pesticides which have similar effects like nicotine as an agonist to nAChRs of insects (Li *et al*., 2011).

Nicotine acts as an insecticide but is also toxic to mammals (Ujvary, 1999). In fact, nicotine has been reported to have a lower lethal dose for rats than flies (Yamamoto, 1999). This spurred a scientific search for compounds that retain the insecticidal properties of nicotine but have selectively less effect on mammals, though initial investigation of nicotine-related compounds (nicotinoids) as insecticides was unsuccessful (Ujvary, 1999). Nevertheless once succeeded the synthetic nicotinoids gain credence as successful broad spectrum insecticide.

Neonicotinoids, like nicotine, are nicotinic acetylcholine receptor agonists. This receptor is normally activated by the neurotransmitter acetylcholine. These receptors are located in both - the central and peripheral nervous systems of mammals but are limited to the CNS in insects; peripheral nervous system of insect is GABAergic not cholinergic. While low to moderate

activation of these receptors causes nervous stimulation, high levels over-stimulate and hence block the receptors (Yamamoto, 1999). This receptor blockage causes paralysis and death. Normally, acetylcholine is broken down by acetylcholinesterase (AChE) to terminate signals to these receptors. However, AChE cannot break down neonicotinoids, and the binding is irreversible. Most neonicotinoids bind much more strongly to insect neuron receptors than to mammal neuron receptors, and hence, these insecticides are selectively more toxic to insects than mammals (Tomizawa, 2004).

Thiacloprid, a cyanamide, shows low affinity for mammalian nicotinic acetylcholine receptors while exhibiting high affinity for insect nAChRs (Tomizawa *et al*., 1999; Yamamoto, 1999). Thiacloprid mimics the action of nicotine in the nervous system, binding at or near the site on the nAChR where nicotine binds, producing an unregulated barrage of nerve impulses, resulting in something akin to a nervous breakdown, and ultimately, death (Tomizawa and Casida, 2003; Tomizawa and Casida, 2005). Molecular difference between thiacloprid and nicotine is presented in Figure A.

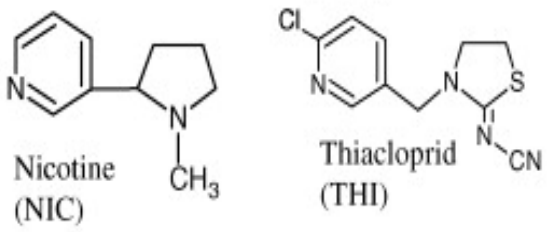


Figure A: Structural difference between nicotine and thiacloprid

Mammals and insects have structural differences in nAChRs, which affect how strongly particular molecules bind, both in the composition of the receptor subunits and the structures of the receptors themselves (Tomizawa *et al*., 1999; Tomizawa, 2004). Nicotine, like the natural ligand acetylcholine, has a positively charged nitrogen (N) atom at physiological pH because it is protonated by water (Yamamoto, 1999; Tomizawa, 2004). Due to positive charge, these compounds have strong affinity towards mammalian nAChRs. At the same time, the charge on nicotine lowers its effectiveness as an insecticide, because the blood–brain barrier prevents free access of ions to the central nervous system, and insect nAChRs are only present in the central nervous system (Yamamoto, 1999; Tomizawa, 2004). The blood-brain barrier does not prevent nicotine poisoning in mammals, because mammalian nAChRs are located in the peripheral nervous system and are necessary for vital functions such as breathing. The low

mammalian toxicity of thiacloprid can be explained in large part by its lack of a charged nitrogen atom at physiological pH. The molecule shows weak affinity to mammalian nAChRs but strong affinity for insect $\alpha_4\beta_2$ nAChRs. Furthermore, the uncharged molecule can penetrate the insect blood-brain barrier, while the human blood-brain barrier filters it (Yamamoto, 1999).

All nAChR molecules contain five subunits, each of which contains a dicysteine loop (Cys-loop) with 13 intervening residues in the N terminal, extracellular domain (Corringer *et al*., 2000; Karlin, 2002).The subunits are classified into α and non-α types according to the presence (α subunits) or absence (non-α subunits) of vicinal cysteine residues in loop C, one of six separate loops (A-F) that make up the acetylcholine (ACh) binding site. Two α and three non-α subunits are recruited to form most heteromeric nAChRs, in which the ligand-binding sites are located at the interfaces of the α and the adjacent non-α subunits (Figure B). The α7, α8, and α9 subunits of vertebrates can form homo-oligomers (Couturier *et al*., 1990), whereas the 10 subunit forms a heteromer with the 9 subunit (Elgoyhen *et al*., 2001). In such cases, the ligand-binding sites are located between two adjacent α subunits (Matsuda *et al*., 2005).

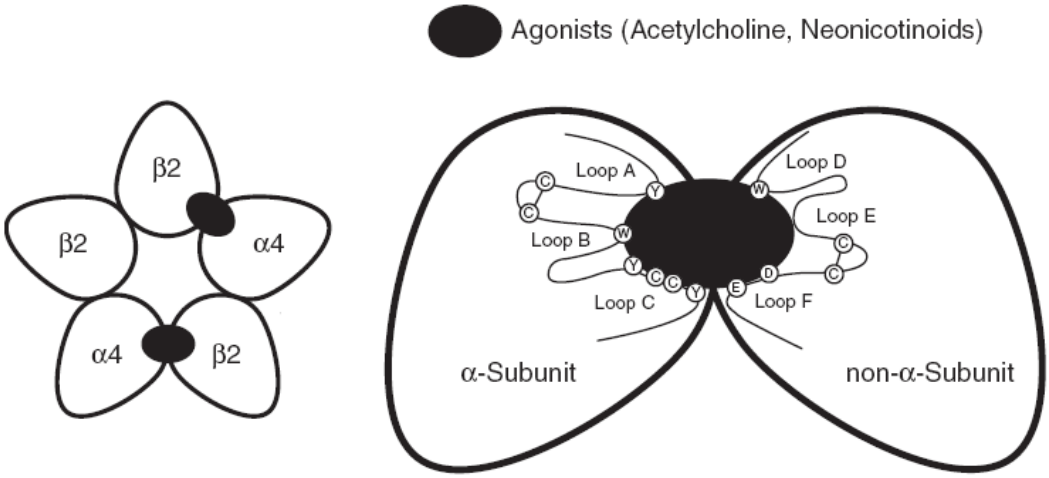


Figure B: Schematic representations of a Nicotinic Acetylcholine Receptor (nAChR) and its ligand binding site (Matsuda *et al*., 2005)

Thiacloprid is specific for $\alpha_4\beta_2$ nAChR of insect CNS. Mammalian central and peripheral nervous system has $\alpha_4\beta_2$ nAChR. Human neuronal nicotinic receptors are pentamers formed by a single alpha subunit (α_7, α_8 or α_9) or a combination of α and β subunits (Ortells and Lunt, 1995; Lindstrom *et al*., 1996). $\alpha_4\beta_2$ nicotinic receptors are abundant in the brain and they account for more than 90 % of the high-affinity nicotine binding sites in the brain (Flores *et al*., 1992; Picciotto *et al*., 1995; Marubio *et al*., 1999). In rat, high affinity nAChR sites

revealed by $[^3H]^{(-)}$-nicotine, are abundant in selective areas of the cerebral cortex (predominantly layers III and IV), thalamus, interpeduncular nucleus and the superior colliculus, but are of low to moderate abundance in the hippocampus and hypothalamus (Clarke *et al*., 1985). The C=N-CN moiety of thiacloprid is hydrolyzed to the amide [C=NC(O)NH2] and also undergoes N–CN cleavage. Descyanothiacloprid is a particularly potent mammalian nAChR agonist (Tomizawa *et al.*, 2000; Klein, 2003).

However, the endogenous agonist acetylcholine is an excitatory neurotransmitter of the cholinergic system. ACh released from the presynaptic memebrane interacts with the binding site located at the extracellular domain of the nAChR/ion-channel complex. A conformational change of the receptor molecule then leads to channel opening, influx of extracellular Na^+ and efflux of intracellular K^+. The nAChR is responsible for rapid neurotransmission in the central nervous system (Matsuda *et al*., 2005). Thiacloprid is not protonated, but the electronegative (δ^-) cyano- tip may bind to a lysine or arginine residue in a subsite of the insect nAChR. Nicotine is protonated at physiological pH and undergoes cation-π interaction with Trp at nAChR subsite in mammal (Figure C). Subsequent to excitation by the endogenous ligand ACh, enzyme acetylcholinesterase hydrolyses acetylcholine and turn off the stimulation. However AChE cannot hydrolyse bound thiacloprid from nAChRs, which results in excessive stimulation of cholinergic receptors (Matsuda *et al*., 2005).

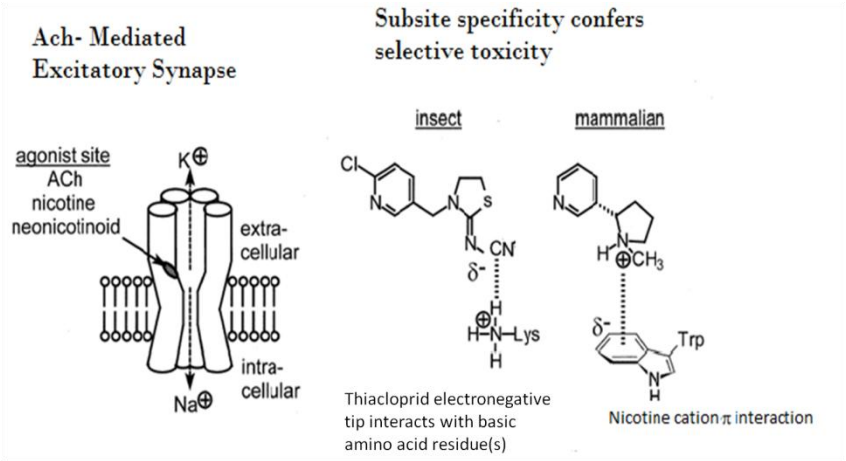


Figure C: Nicotinic receptor-ionophore complex in which subsite specificity confers selective toxicity of neonicotinoids (thiacloprid) for insects and nicotinoids (nicotine) for mammals (adapted from Matsuda *et al*., 2005).

Thiacloprid poisoning causes increased stimulation at all three locations of nAChR: (1) the neuromuscular junction, (2) the autonomic ganglia and (3) the CNS. At the neuromuscular junction, overstimulation of nAChRs results first in muscle fasciculation that ultimately results

in weakness or even paralysis (Barthold and Schier, 2005). The weakness is probably due to desensitisation of the receptor. At the ganglia, excessive stimulation causes activation of the autonomic nervous system (Sheridan *et al*., 2005).

The main symptoms of thiacloprid toxicity are related to the excessive stimulation of the cholingergic receptors at neuronal, ganglionic and muscular interfaces. These symptoms include muscle fasciculation and weakness, papillary dilatation, ptosis and irregular respiration (Sheridan *et al*., 2005; Hassel, 2006). CNS symptoms are also observed, notable among which are giddiness, ataxia and seizures (Barthold and Schier, 2005). The cholinergic input to various brain nuclei gives rise to increased glutamatergic and GABAergic activity and causes brain seizures. The seizures progress rapidly to 'status epilepticus', which may lead to profound structural brain damage. The seizures and resulting brain damage are initiated by cholinergic over-stimulation which triggers seizures in susceptible brain regions. The seizures cause the release of excessive amounts of glutamate from affected neurons and the released glutamate gives rise to excitotoxicity and cell death (Solberg and Belkin, 1997).

Conventionally the extent of neurotoxicity is measured through the estimation of relevant biochemical markers. AChE activity is often estimated to measure the acute neurotoxicity however, delayed neuropathy is assessed by estimating the neuropathy target esterase (NTE, also called as neurotoxic esterase) (Ehrich *et al*., 1997). Neuropathy target esterase plays critical roles in embryonic development and maintenance of peripheral axons. NTE *in vivo* is poorly defined. It is known to hydrolyze lysophosphatidylcholine (LPC) *in vitro* and may protect cell membranes from cytotoxic accumulation (Vose *et al*., 2008). NTE mutations are associated with progressive upper and lower-motor neuron disease indicating the importance of NTE in maintaining the integrity of corticospinal tract and peripheral motor axons (Shirley *et al*., 2008). NTE-LysoPLA inhibition is known to lead to localized accumulation of lysolecithin, a known demyelinating agent and receptor-mediated signal transducer (Quistad *et al*., 2003).

NTE is anchored to the cytoplasmic face of the endoplasmic reticulum membrane and is particularly abundant in neurons, the placenta, and the kidney (Glynn, *et al*., 1998; Li *et al*., 2003; Moser *et al*., 2004; Zaccheo *et al*., 2004). Loss of NTE activity results in abnormally elevated levels of phosphatidylcholine in brain and impairment of the constitutive secretory pathway in neurons. Mouse or human neuroblastoma cell-lines can be considered useful *in*

vitro models to distinguish esterase-inhibiting neurotoxicants. NTE esterase domain accelerates the elongation of neurite processes in human neuroblastoma (SK-N-SH) cell line (Ping-An *et al*., 2005).

Thiacloprid being a neonicotinoid insecticide mainly targets the nervous system by mimicking the neurotransmitter acetylcholine and binds with nAChRs. Few studies have been performed in mammals regarding the toxic effects of neonicotinoids. Further, these studies have also mostly focussed on the mechanism of neurotoxicity of imidacloprid, a nitroimine derivative. Nevertheless, thiacloprid, a cyanamide though a frequently used agrochemical and a potent neurotoxicant like imidacloprid, there has been very little focus on its neurotoxic effects on a non-target organism like mammals.

Hence, it was thought pertinent to assess the possible esterase-dependent neuropathy induced by thiacloprid in the non-target mammalian system. This was done through estimating blood and plasma activities of esterases (AChE and NTE) and also by carrying out AChE localization in different regions of the brain. The study was also extended to explore any possible relation between NTE and neuronal damage, a likely toxic effect of thiacloprid. Neuronal damage was observed by Nissl staining on fresh frozen brain sections using cresyl violet stain. Further, inhibition of esterase may lead to development of paralysis or ataxia which is a known neurotoxic mechanism of pesticides. Hence, a rotarod test was also conducted to assess the neuromuscular co-ordination in thiacloprid intoxicated rats. Further, delayed neuropathy could reflect as possible development of demyelination which was explored through transmission electron microscopy of nerve tissues. Further, an *in vitro* study using IMR-32 cell line was also conducted to analyze the possible toxic effect of thiacloprid on the growth and elongation of neurite.

MATERIAL AND METHODS

Male Sprague-Dawley rats procured from a CPCSEA approved animal breeder (SPARC Ltd., Baroda) were used in all experiments. All experiments were carried out in accordance with protocols approved by IAEC according to CPCSEA guidelines, and all efforts were made to minimize the number of animals used and to take care that they were subjected to minimal suffering. Rats were kept in the departmental animal house (827/ac/04/CPCSEA) at controlled temperature (24 ± 2°C) and light-dark schedule (12:12) and were provided laboratory rat food (Pranav Agrochemicals) and water *ad libitum*. Thiacloprid (Allanto, 21.7%SC) was procured

from local market and diluted in distilled water to make the desired dose concentration. Experimental animals were grouped into three as control, low dose and high dose group, with five animals in each group for subacute as well as subchronic oral administration.

At the end of experiment, rats were sacrificed after overnight fasting. Blood was collected in EDTA vacutainer for cholinesterase activity. Target organ was removed and fixed in 10% buffered formalin. For transmission electron microscopy (TEM) 4% paraformaldehyde was used for tissue fixation.

Protocol I: Assessment of Esterase

Acetylcholinesterase (AChE): Acetylcholinesterase activity was performed by the method of Ellman *et al*. (1961). Activity of AChE was measured in whole blood, plasma, various regions of brain and neuroblastoma IMR cell line. The assay uses acetylthiocholine as a substrate. AChE hydrolyses the acetylthiocholine to produce thiocholine which in turn reduces DTNB liberating nitrobenzoate, which absorbs at 412nm.

Neurotoxic esterase (NTE): The procedure is a modification for rat brain of a standard method for hen brain (Johnson, 1977) and for IMR cell line (Correll and Ehrich, 1991). Phenyl valerate was used as a substrate and phenol liberated by NTE was measured at 490nm.

Localization of AChE: Direct colouring method described by Karnovsky and Roots (1964) was adapted for the localization of acetylcholinesterase in fresh frozen sections of brain.

Protocol II: Functional observation battery (FOB)

Forced swimming test (FST) was performed according to the protocol of Cryan *et al*. (2002). The rats were forced to swim for 10 minutes in the glass cylinders containing water. This activity was repeated every month. Parameters like active swimming, floating and dipping were recorded with time.

Rotarod test was used to assess motor coordination and balance in treated rats. Time (latency) taken by the rats to fall off the rotating rod at different speeds or under continuous acceleration (e.g. from 4 to 40rpm) was recorded. Each animal was observed for 5 minutes and each fall was noted.

Protocol III: Assessment of neuronal damage

Neuronal damage was assessed by cresyl violet staining. 15µm thick cryosections were stained with cresyl violet and observed under microscope (Leica DM2500). Demyelination was observed using Transmission electron microscopy (TEM) (Glauert, 1974). Tissues were fixed in 2.5% glutaraldehyde for 24 hrs at 4ºC. The tissues were rinsed with buffer and osmicated in 2% osmium tetroxide. Tissues were rinsed again and dehydrated using increasing alcohol concentrations, followed by rinsing in propylene oxide. The specimen was put in a mixture of 50% propylene oxide and 50% resin for at least 2 hours and then in 100% resin as embedding medium. Resin blocks were prepared. Samples were cut on an ultra microtome and sections were stained with uranyl acetate for 20 minutes and then rinsed. Sections were observed under TEM for myelin pattern analysis.

Protocol IV: Histopathological evaluation

Brain tissue was fixed in 10% buffered formalin for 24hrs and then dehydrated in alcohol. After clearing in xylene, paraffin block was prepared. Sectioning was done using microtome and sections were stained with haematoxylin and eosin and observed under the microscope (Leica DM2500).

Protocol V: *In vitro* study on neuroblastoma cell line

IMR 32 human neuroblastoma cell line was used to study the effect of thiacloprid on neurite growth and process elongation. The cell line was procured from National Chemical Laboratory, Cell culture facility, Pune. Culture was maintained as an adherent cell line in T25 flask by providing Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 100µl of antimycotic solution at 37°C in a 5% CO_2: 95% air-humidified atmosphere. IC50 value was calculated through MTT assay. Cell morphology was observed and esterase activity was also estimated.

Statistical analysis

Data are expressed as a Mean ± SE. Statistical analysis was performed using One-way ANOVA followed by post hoc Bonferroni test for multiple comparisons using SPSS ver.12.0. The p values of ≤ 0.05 were considered statistically significant.

RESULTS

The effect of repeated exposure to thiacloprid during the subacute and subchronic studies on acetylcholinesterase (AChE) activity in blood, plasma and brain is shown in Table 3.1. The results indicate that activity of AChE was higher in blood and plasma in the thiacloprid treatment groups in the subacute study whereas the same was observed to be low in the subchronic study as compared to control values. However, these differences were not statistically significant. Brain AChE activity in animals treated at the dose of 100mg/kg body weight in subchronic study was found to be significantly decreased compared to control rats ($p \leq 0.05$). Similar trend was observed for animals given a lower dose during subchronic study and higher dose during subacute study, but the differences were not statistically significant (Figures 3.1 and 3.2). Moreover, results of AChE localization also indicated much less intensely localized AChE in thiacloprid treated rat brain as compared to control rat brain (Figure 3.9).

Table 3.2 shows values for neuropathy target esterase (NTE) activity in brain of both the thiacloprid dosage groups for both the study periods. Activity of NTE was observed to be lower in both the treatment groups during both the subacute and subchronic studies as compared to control values. Statistically significant decrease ($p \leq 0.05$**)** in brain NTE activity was observed for rats dosed at 100mg/kg body weight in the subchronic study (Figure 3.3).

Neuromuscular coordination of animals was observed using rotarod test after 90 days, results of which are presented in Table 3.3. When rats were kept on the rotarod moving at 15rpm, the time taken to fall off the rod was not significantly different between the control and treatment groups. However, with increasing speeds of rotation, treated rats were observed to fall in less time as compared to control rats. At 20 rpm, time recorded for rats falling off the rotarod was significantly smaller ($p \leq 0.05$) for the high dose treatment group as compared to control rats. Similary at 25 rpm, both the treatment groups experienced much difficulty in staying on the rotarod and fell off the rod significantly ($p \leq 0.05$) earlier (Figure 3.4).

Data compiled in Table 3.4 shows the performance of experimental animals in forced swimming test. Values represent the time duration in seconds, taken by the animals to become inactive (floating) or immobile (immobility time). Swimming test was done before intoxication of thiacloprid, values for which are given as on day '0' and then thiacloprid was administered to rats for 28 and 90 days. On day 0, there was no significant difference in the values of the immobility time (Figure 3.5). After 28 days of intoxication of test compound, animals of low

dose group were observed to attain immobility earlier as compared to control and those of high dose group also showed the same trend as the low dose group, with the difference being statistically significant as compared to control ($p \leq 0.01$). 90 days of repeated thiacloprid doses of 50mg/kg body weight and 100mg/kg body weight caused the animals to become inactive significantly earlier as compared to reference group of rats ($p \leq 0.05$ for low dose group and $p \leq 0.01$ for high dose group).

Figure 3.11 shows results of cresyl violet staining of brain. Control brain sections showed nerves with prominent nuclei. Hippocampal region of brain also showed more intense staining of cresyl violet, indicating greater neuronal cell density (Figure 3.11A). In the treatment groups, at the end of 90 days of study period, the area of the cerebral cortex was observed to be decreased compared to control as revealed by the cresyl violet staining of treated rat brain of high dose group. Neuronal cell density was observed to be decreased as compared to the control brain and clear pyknosis was also observed in brain sections of the treatment group in the subchronic study (Figure 3.11B). Further investigation of neuronal damage was done by transmission electron microscopy to assess demyelination and axonal loss in treated animals. The demyelinated nerve in thiacloprid intoxicated CNS and sciatic nerve showed extensive loss of myelin around axon, hypomyelination and vacuolar degeneration (Figure 3.12C-3.12F), whereas the neurons in the control group were found to have intact myelin sheath (Figure 3.12A-3.12B).

Histopathological evaluation of the brain of the control animals revealed intensely stained neurons in cerebrocortex area and more granules in granular area of hippocampus and thalamus in rat brain (Figure 3.13 I&II), whereas repeated exposure of thiacloprid for 90 days developed axonal swelling, vacuolar degeneration, spongiform changes and pyknotic nuclei in neuron in treated rat brain (Figure 3.14 V-VIII). Minimal haemorrhages, vacuolar degeneration and mild gliosis with spongiform of changes were profoundly observed in the brain of treated rat (Figure 3.13 III&IV).

Figure 3.6 shows percent inhibition of IMR 32 neuroblastoma cell line exposed with thiacloprid. MTT assay was performed while assessing thiacloprid cytotoxicity for IC50 value. The calculated IC50 value of thiacloprid for IMR 32 cell line is 7.02±1.3µg/ml (27µM). Esterase activity of the cells was also assessed by exposing them to 0.35µg/ml and 0.7µg/ml thiacloprid for different time intervals. AChE activity was found to gradually decrease with

increasing time of thicacloprid exposure and concentration. Statistically significant decrease in activity was observed at 60 hrs in cells given 0.35µg/ml and 0.7µg/ml of thiacloprid exposure ($p \leq 0.05$) Table 3.5, Figure 3.7). Similar trend of decreased activity with increased time of exposure and concentration of test compound was also observed for NTE activity, however, decrease in activity was only observed to be significant with 0.7ug/ml of exposure for 60 hrs ($p \leq 0.05$) (Table 3.5, Figure 3.8).

Figure 3.15 show acridine orange-ethidium bromide staining of IMR 32 cells. Figure 3.15B shows that thiacloprid-exposed cells experienced greater degree of cell-death as compared to control cells. Cells emitting green fluorescence are viable cells and those emitting red fluorescence are necrotic cells. Moreover, the test compound treated cells were observed to have less neurite processes, due to which proper growth of these cells could not have been achieved. Due to decreased elongation of processes, the communication between cells was also diminished as compared to that in control cells (Figure 3.16 A&B).

DISCUSSION

The current study was aimed at finding the possible neurotoxic effects of the neonicotinoid thiacloprid on the mammalian system. One of the many analyses carried out in this regard was assessing the acetylcholinesterase activity in thiacloprid intoxicated rats. It was observed that rats given subacute exposure to thiacloprid showed increased AChE activity in blood and plasma and decreased brain AChE activity, even though the changes were not statistically significant. Thiacloprid works as an agonist to nAChR and binds with it, stimulating the nerve. This nerve activation, it is proposed, induces a signal to release the substrate from the receptor and hence, AChE levels tend to elevate to cleave the substrate from the receptors of stimulated nerve (Kimura-Kuroda *et al*., 2012). This probably may be the reason for the observed increase in AChE activity in blood and plasma of treated rats. However, activity of AChE in brain of treated rats was found to be decreased in the 28 days study (not significant) and also in 90 days study (significant decrease). In similar studies done by Rodrigues *et al*. (2010), decreased activity of acetylcholinesterase in rat brain and decreased choline uptake in synaptosomes from hippocampus area of rat brain treated with cyanogroup of neonicotinoid thiamethoxam was observed. Bhardwaj *et al*. (2010) also reported significant decreased activity of AChE in the brain of female rats given 90 days repeated exposure of imidacloprid at a dose of 20mg/kg body weight. Chao and Casida (1997) reported the accumulation of imidacloprid in mouse brain following direct intra-peritoneal administration. However, Brunet *et al.* (2004) have also

reported that neonicotinoid is highly absorbed in human intestinal cells, suggesting its potential anticholinergic effects. The cause of this inhibition is unknown because neonicotinoid is not ChE inhibitor, since plasma AChE is synthesized in the liver, the decrease in plasma AChE activity may be related to observed changes in liver function (EPA, 2006a). Results of the current study were also supported with localization of AChE in frozen sections of brain, which was observed to be much less intense in thiacloprid treated rat brain as compared to control. This pattern was more prominent in high dose treatment group of experimental animals in the subchronic study. Similar localization studies related to the toxic effects of neonicotinoids have not been reported.

Neurotoxic target esterase (NTE) activity in rat brain was also analyzed in present study. Activity of NTE was observed to be decreased in treatment group of rats in both 28 and 90 days studies. However, decrease was significant only for animals of high dose treatment group in the subchronic study. Similar studies about thiacloprid or any other neonicotinoid pesticide have not been reported till date. Nevertheless, the above results could emerge as a pertinent reason for observed neuronal damage in thiacloprid intoxicated rats.

In this study, rats exposed to thiacloprid for 28 days and 90 days were observed to attain immobility earlier as compared to control rats in the forced swimming test (FST), an indication of lesser coordination in movement of legs. The attainment of early immobility caused by thiacloprid in the FST can be attributed to a locomotors effect caused by this test compound, since there was a change in total locomotion after thiacloprid administration. During the FST of rats given 90 days of exposure to the test compound, it was observed that the rats started dipping in water in much less time as compared to control and eventually became rather inactive (floating) and were unable to make an effort to swim. In addition to the inactivity observed during the FST, it was also observed that thiacloprid treated rats developed a dragging locomotion and even poking the animal with a blunt object did not cause the animal to respond and move further. This observation has been video-graphed. Such a significant decrease in locomotor activity in the rats treated for 90 days with thiacloprid could be an indication of the accumulation of thiacloprid or its metabolites in the brain. This discussed notion does not find any support from any report published with regard to thiacloprid. However, it has been reported that oral exposure of neonicotinoid imidacloprid for 90 days at different doses in female rats resulted in significant alteration in various aspects of spontaneous locomotor activity. A significant decrease in distance travelled, ambulatory time

and stereotypic time was noted after 90 days in animals exposed with 20 mg/kg/d imidacloprid (Bhardwaj *et al*., 2010).

Hamm *et al*. (1994) had suggested that compared to other behavioural tests, the rotarod test is a more sensitive and efficient index for assessing motor impairment produced by brain injury. The present study on rats exposed to thiacloprid for 90 days showed decreased neuromuscular coordination which was reflected as increasing number of falls from the rotating rod as compared to the control animals. It was found that thiacloprid treated rats could not keep balance on the rod at 25 rpm. This observation could be co-related to the development of ataxia in thiacloprid exposed rats in the subchronic study as mentioned earlier. Significant sensorimotor impairments similar to the current results have been reported in female rats after exposure to a single dose of imidacloprid (Abou-Donia *et al*., 2008). The motor and behavioural alterations reported here could also be related to alterations in the brain chemistry of animals treated with thiacloprid. Development of ataxia in rats could be a cummulative result of thiacloprid intoxication effects like neuronal damage, thyrotoxicosis and lack of neuromuscular coordination which lead to dysdiadochokinesia observed in the rotarod test. Barnard *et al*. (1971) have even suggested a preclinical picture of cerebeller syndrome with dominant ataxia of gait and less ataxia of the limbs occuring in patients with hypothyroidism.
A sensorimotor disorder associated with depression and characterized by a strong urge to move the legs associated with paresthesias and motor restlessness has also been described in patients with chronic kidney disease caused by the medication used which proved to be neuropathic (Szentkiralyi *et al*., 2009; Cavallini *et al*., 2010). It is possible that thiacloprid could possibly act as a depressant also. Tomizawa (2004) suggested that stimulation of the nervous system by acute or sustained exposure to neonicotinoid chemicals may lead to synaptic plasticity or attenuated neuronal functions.

It is reported that such neurobehavioral deficits may reflect dysfunction at multiple anatomical areas in the central nervous system. Brain injury or damage may result into abnormal sensorimotor coordination (Hamm *et al*., 1994). With this notion, neuronal damage was assessed in the current study by cresyl violet staining. Cresyl violet stains Nissl bodies of neuron. Nissl bodies represent aggregations of rough endoplasmic reticulum, containing numerous ribosomes involved in the synthesis of neurotransmitter such as acetylcholine (Bear *et al*., 2007). Hippocampus area of brain of thiacloprid treated rat exhibited pyknosis and less density of neurons as compared to control rats when stained by cresyl violet indicating

neuronal damage. *In vivo* studies in rat as well as *in vitro* studies have suggested links between decreased hippocampal neurogenesis and depression (Malberg *et al*., 2000; Manev *et al*., 2001), which support the behavioural observations noted in the present study. Trauth *et al*. (1999) also reported evidence supporting hippocampal cell damage in female rats exposed to nicotine. The inhibitory effect on AChE activity due to thiacloprid intoxication can be correlated with results of neuronal cresyl violet staining, which stains RNA (Nissl body involved in acetylcholine synthesis).

Further, neuropathy target esterase (NTE) activity was also estimated in the brain of the experimental animals. NTE is an integral membrane protein in vertebrate neurons (Glynn, 1999) and it plays an important role in neural development, possibly via involvement in a signalling pathway between neurons and glial cells. In the current study, NTE activity was found to be decreased in the thiacloprid treated rats indicating neuronal damage of the brain in these animals. This decrease was significant, as compared to control animals, for the animals given subchronic exposure to the test compound. Vacuolated myelin degeneration of neuron was observed in TEM images. NTE inhibition is also related to localized accumulation of lysolecithin, a known demyelinating agent and receptor-mediated signal transducer (Quistad *et al*., 2003). In the present study, the myelination status of the nervous tissue was also assessed in the experimental animals. As expected, the thickness of myelin was found less in the treated group of animals compared to control neurons. It is reported that on exposure to organophosphorous, negatively charged phosphate group attaches to the active site of serine, triggers demyelination by inhibiting catalytic activity of NTE and AChE (Lotti and Moretto, 2005). Researchers have suggested that myelin degradation is induced via Ca^{2+} influx into myelin and subsequent activation of cytosolic phospholypase A_2 and calain, which break down the myelin lipids and protein and ultimately lead to excessive stimulation of Ca^{2+}-dependent degradative pathways (Fu *et al*., 2007; Trapp and Stys, 2009). Significant excitatory Ca^{2+} influxes have been reported to be evoked by acetaminophen, imidacloprid and nicotine at concentrations greater than 1μM in small neurons in cerebellar cultures that expressed the mRNA of the α3, α4, and α7 nAChR subunit (Zoli *et al.,* 1995; Kimura-Kuroda *et al*., 2012). Hypothetically speaking, the reasons discussed above may also probably be true for the neurotoxic mechanism of thiacloprid in causing demyelination of neurons.

The pattern of myelin degeneration observed in the treatment group in the present study is comparable to Alexander disease, a foetal neurodegenerative disease in which dysplasia of

myelin occurs as a vacuolar pattern which leads to neuronal degeneration in humans (Roessmann *et al*., 1980). Neuronal degeneration in this disease is due to the lack of glial fibrillar acidic protein (GFAP). GFAP is proposed to play a role in cell-cell communication and is also known to modulate phosphorylation at various serine or threonine residues by PKC and PKA which are two kinases important for the cytoplasmic transduction of signals (Tuccari *et al*., 1986). This mechanism may also be one possibility in the present study, causing inhibition of AChE and axonal degeneration by inhibition of GFAP. It has also been reported that loss of GFAP in nerve impairs Schwann cell proliferation and delays nerve regeneration after damage. In many peripheral neuropathies, axonal loss causes disabling and permanent deficits and may result from inefficient nerve regeneration due to a defective relationship between Schwann cells, axons and the extracellular matrix. These interactions are mediated by surface receptors and transduced by cytoskeletal molecules (Triolo *et al*., 2006).

AChE inhibition and NTE inhibition due to thiacloprid exposure induced histopathological changes in brain as could be concluded from the histological observations made for rat brain. Axonal swelling, pyknotic nuclei brain cells with loss of granules in granular layer of hippocampus and thalamus region of brain were the several pathological changes observed in the brain of treatment group of animals. In the absence of published reports it could be hypothesized that demyelination may be the result of axonal swelling which in turn disturbs the integrity of myelin sheath. Mild focal vacuolar degeneration and gliosis with spongiform change was also observed in treated rat cerebrum. Gliosis is a nonspecific reactive change of glial cells in response to damage to the central nervous system (CNS) and the process of gliosis involves a series of cellular and molecular events that occur over several days (Fawcett and Asher, 1999). Proliferation of astrocytes is increased in gliosis which accompanies traumatic brain injury as well as many neuropathologies (Zhang *et al.*, 2010; Rivera-Zengotita and Yachnis, 2012). Microglia is one of the glial cells which is increased during gliosis and induce the release of neurotoxic factors that promote increased degeneration of the neuron and more rapid phagocytosis by the microglia (Streit *et al.*, 1999). Oligodendrocytes are another type of glial cell which generate and maintain the formation of myelin around the axons of large neurons in the CNS, allowing for rapid transmission of neural signals. Unlike astrocytes and microglia, oligodendrocytes undergo a much more limited reaction to injury (Rivera-Zengotita and Yachnis, 2012).

The degeneration of axons as a result of trauma or pathology invariably results in the degeneration of the myelin sheath (Fawcett and Asher, 1999). Earlier studies have shown that concentration of pesticides and metabolites in plasma and brain generally correlate with the severity of toxicity and symptoms of neurotoxicity which are found to increase with the pesticide concentration in brain (Nagata *et al*., 1996). The histopathological changes observed in the brain of treated rats in the current study also provide support to the neurobehavioural effects observed earlier indicating accumulation of thiacloprid and its metabolites in the brain (although kinetic studies of thiacloprid have not been done). Pathological alteration due to the exposure of imidacloprid for the period of 90 days has also been observed in rat by Bhardwaj *et al*. (2010). Nicotine exposure to brain tissue reportedly leads to nAChR induced apoptotic cell death in hippocampal area of brain (Berger *et al*., 1998). Carlson *et al*. (2000) have also observed nicotine induced axonal degeneration in brain caused due to several days of its administration.

Moreover, it is well documented that transient but significant expression of nAChRs during the perinatal stage is important for brain development (Role and Berg, 1996; Dwyer *et al*., 2009). In the developing brain, $\alpha_4\beta_2$ and α_7 subtypes of the nAChR have been implicated in neuronal proliferation, apoptosis, migration, differentiation, synapse formation, and neural-circuit formation. Due to nicotine-like mechanism of neonicotinoids, they are likely to affect these important processes when they activate nAChRs (Role and Berg, 1996; Dwyer *et al*., 2009). *In vitro* effects related to thiacloprid exposure on a human cell line may be useful in predicting *in vivo* alteration in the animal model. Thus, an *in vitro* study was also performed to observe the neuronal growth-related damage due to thiacloprid exposure, which can be useful in understanding observations made in the *in vivo* study and can also be correlated with the brain development process of neonates. For this study, neuroblastoma IMR 32 cell line was selected because neuroblastoma is a childhood solid tumor composed of primitive cells derived from precursors of the autonomic nervous system. This neoplasm has the highest rate of spontaneous regression of all cancer types and has been noted to undergo spontaneous and chemically induced differentiation into elements resembling mature nervous tissue. As such, neuroblastoma has been a prime model system for the study of neuronal differentiation (Abemayor and Sidell, 1989).

In the present study, thiacloprid-induced percent cell-inhibition concentration (IC50) was measured to be 7.02±1.3µg/ml (27µM) in human neuroblastoma cell line using MTT assay.

Tomizawa and Casida (2000) have also reported a nearby IC50 value in mouse fibroblast M1 cell line. IC50 value reported for imidacloprid was 70µM and that for thiacloprid was 19µM in fibroblast M1 cells. Results given by Tomizawa and Casida (2000) suggested that thiacloprid is a more potent toxicant than imidacloprid. It is also mentioned in this study that the descyano metabolite was more potent than the parental thiacloprid for M1 cells. In metabolically active cells, MTT is reduced by mitochondrial succinate dehydrogenase to give a dark purple-coloured product (Mosmann, 1983). According to the calculated IC50 value of thiacloprid obtained in the present study, it can be concluded that thiacloprid can lead to mitochondrial dysfunction at very low dosage and develop neurotoxicity by disturbing the developing neurons.

Subsequently, we estimated the esterase activity in IMR 32 cells exposed to $1/10^{th}$ (0.35µg/ml) and $1/20^{th}$ (0.7µg/ml) of IC50 value of thiacloprid for different time intervals. Thiacloprid exposure for 60 hours significantly inhibited the activity of acetylcholinesterase in both the studies of different concentrations of exposure, indicating that within 60hrs thiacloprid could bind with nAChR and inhibit the choline uptake from the synaptosome of developing cells. NTE activity was also observed to be significantly low in these cells on 60hrs of exposure at a concentration of 0.7µg of thiacloprid. The neonicotinoid imidacloprid has also been reported to act as an agonist or an antagonist of nAChRs at 10µM in rat pheochromocytoma (PC12) cells (Nagata *et al*., 1998) and to change the membrane properties of neurons at ≥10µM in the mouse cochlear nucleus (Bal *et al*., 2010).

IMR cells exposed to these different thiacloprid concentrations were also observed for the neurite growth. At higher concentration we observed that neurite growth was inhibited and elongation of cell processes was very less and not properly attained among the cultured cells which may also probably disturb communication between these cells. Moreover, acridine orange and ethidium bromide staining of the cells evidently showed more cell death in thiacloprid exposed cells as compared to control cells. The dead cells emitted red fluorescence by taking ethidium bromide while live cells were stained with acridine orange and gave green fluorescence. These observations being the first of its kind could not be supported with reports on any studies related to such pesticides. However, recent studies have reported that gestational nicotine exposure modulates the cell-adhesion and cell-death/survival systems in the brains of adolescent rats and may lead to numerous behavioural and physiological deficits (Cao *et al*., 2011; Wei *et al*., 2011).

CONCLUSION

Administration of thiacloprid caused adverse motor and behavioural alterations. Based on the results of the behavioural tests, it can be believed that thiacloprid may act as a depressant. The current study is the first to report that thiacloprid has enough potential to cause neuronal damage and inhibit the acetylcholinesterase and neuropathy target esterase activities. Cresyl violet staining of the brain tissue gave clear indication about the hippocampus neuronal pyknosis and damage as well as less neuronal density in brain caused by thiacloprid administration. Thiacloprid was also found to be a potent toxicant for growing neuronal cells as could be established from results of the *in vitro* study. The neuroblastoma IMR 32 cell growth inhibition could be concluded from observations of disruptions in neurite elongation and cell communication. Based on the results of the *in vitro* study, it is possible that thiacloprid has the potential to damage the developing foetus. Thyroid hormone is known to play a role in normal brain development and it has already been observed that thiacloprid also results in development of thyrotoxicosis (Chapter 2). Further, the pathological changes in brain and specific AChE and NTE inhibition observed in the present study suggest that multiple brain region abnormalities may be involved in disruption of neuromuscular coordination, which is reflected as abnormal locomotor activity. Therefore, we can conclude that thiacloprid may potentially affect human health adversely, especially the developing brain. Moreover, such a toxicant that affects AChE levels or acts as agonist or antagonist at the nicotinic ACh receptors may negatively influence the latent processes of children's neurological function.

Table 3.1. Effect of thiacloprid administration on acetylcholinesterase activities in various tissues of SD rats

Group	Subacute (µM ATC/min/dl)			Subchronic (µM ATC/min/dl)		
	Plasma	Blood	Brain	Plasma	Blood	Brain
Control	0.63±0.09[@]	0.68±0.1	0.66±0.1	0.94±0.06	0.77±0.07	1.01±0.1
Low dose	0.58±0.1	0.84±0.12	0.66±0.12	0.64±0.11	0.58±0.12	0.61±0.1
High dose	0.67±0.1	1.1±0.29	0.63±0.13	0.61±0.13	0.59±0.14	0.55±0.11↓*

Table 3.2. Neurotoxic esterase activity in the brain of thiacloprid intoxicated SD rats

Group	Control	Low dose	High dose
Subacute	2.6±0.13[@]	2.1±0.16	2.0±0.25
Subchronic	2.4±0.11	1.9±0.14	1.8±0.16↓*

Table 3.3. Effect of 90 days exposure of thiacloprid on neuromuscular coordination using rotarod test

Group	Time (Latency) in minute		
	15 rpm	20 rpm	25 rpm
Control	2.4±0.03[@]	2.4±0.04	2.3±0.05
Low dose	2.3±0.07	2.1±0.13	1.9±0.15↓*
High dose	2.1±0.15	1.9±0.14*	1.7±0.15↓*

Values are expressed as Mean±SE; n=5 for each group; *p≤0.05; ** p≤0.01

Table 3.4. Time to float during the forced swimming test in control and thiacloprid treated rats

Group N=5	Immobility time (In second)		
	Control	Low dose	High dose
0 day	64.8±0.9[@]	65.2±0.8	65.6±0.8
28 day	65.2±0.8	62.2±1.0	59.6±1.2↓**
90 day	66.4±0.9	57.4±1.9↓*	54.0±3.1↓**

Table 3.5. Effect of thiacloprid on esterase activity in proliferative neuroblastoma cells at different time interval

Enzyme activity	Hours of exposure			
	24hrs	36hrs	48hrs	60hrs
AChE				
Control	0.18±0.007[@]	0.18±0.008	0.19±0.01	0.21±0.01
0.35µg	0.18±0.008	0.15±0.013	0.14±0.012	0.13±0.013↓*
0.7 µg	0.15±0.008	0.14±0.01	0.11±0.014	0.1±0.015↓*
NTE				
Control	3.2±0.12[@]	3.3±0.12	3.3±0.11	3.4±0.13
0.35µg	3.3±0.17	3.1±0.17	2.9±0.1	2.7±0.18
0.7 µg	3.2±0.12	2.9±0.14	2.9±0.12	2.5±0.13↓*

[@]Values are expressed as Mean ± SEM; n=5 for each group; *p≤0.05; ** p≤0.01

Figure 3.1. Effect of subacute thiacloprid exposure on AChE activity in rat

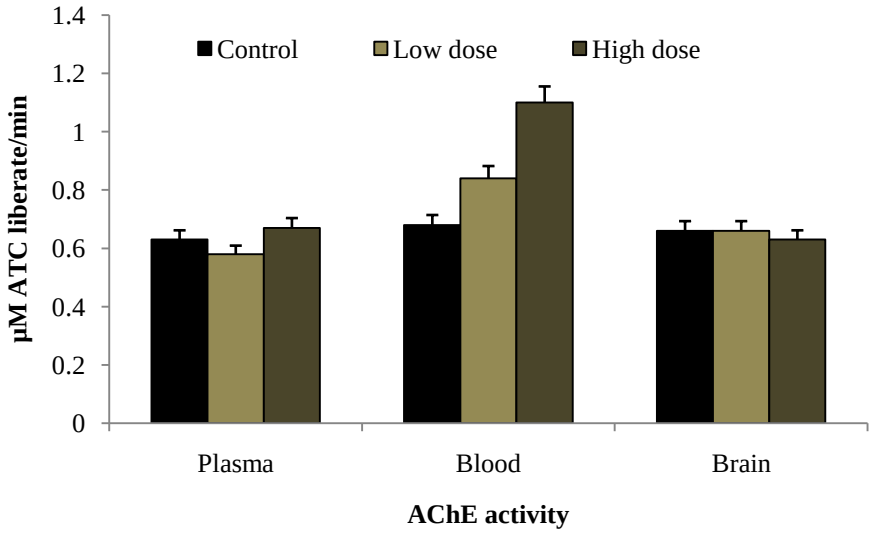


Figure 3.2. Effect of subchronic thiacloprid exposure on AChE activity in rat

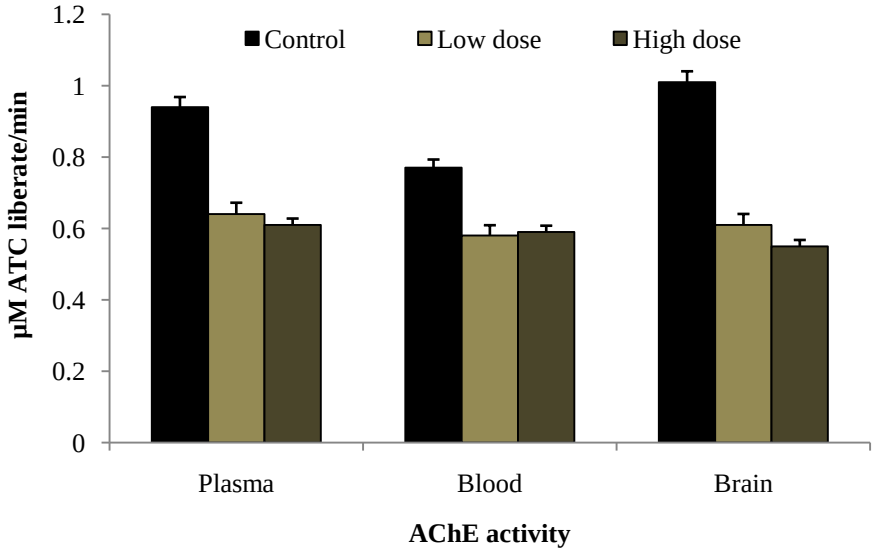


Figure 3.3. Effect of thiacloprid exposure on NTE activity in rat brain

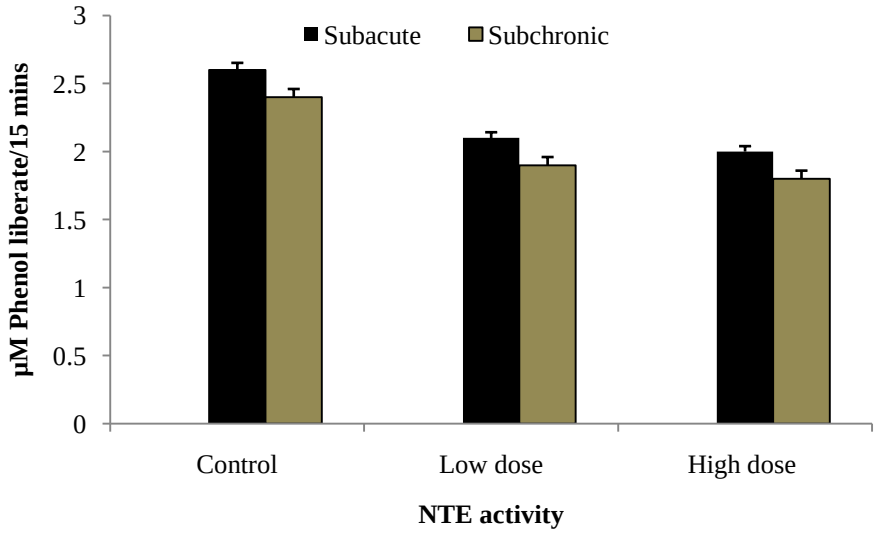


Figure 3.4. Effect of thiacloprid on neuromuscular coordination and grip strength

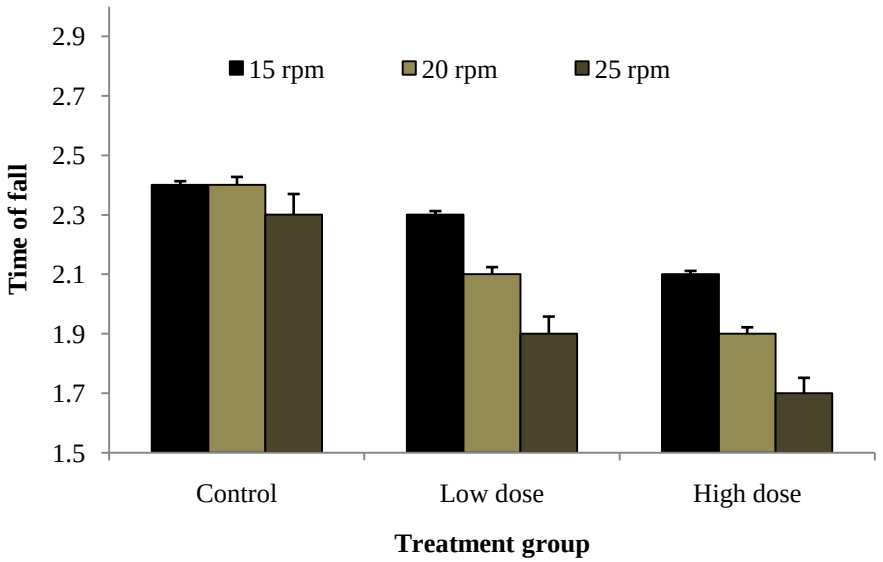


Figure 3.5. Immobility time recorded for animals of various groups during forced swimming test

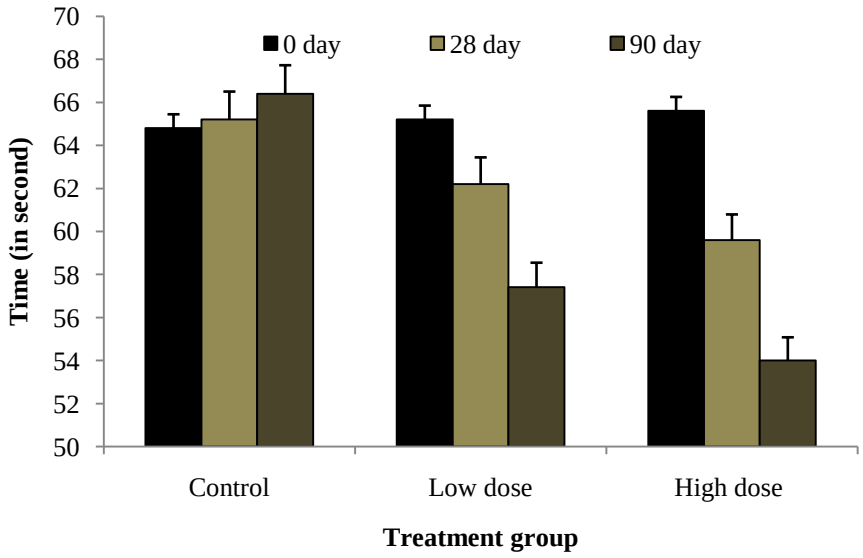


Figure 3.6. Effects of thiacloprid on cell viability of IMR cells

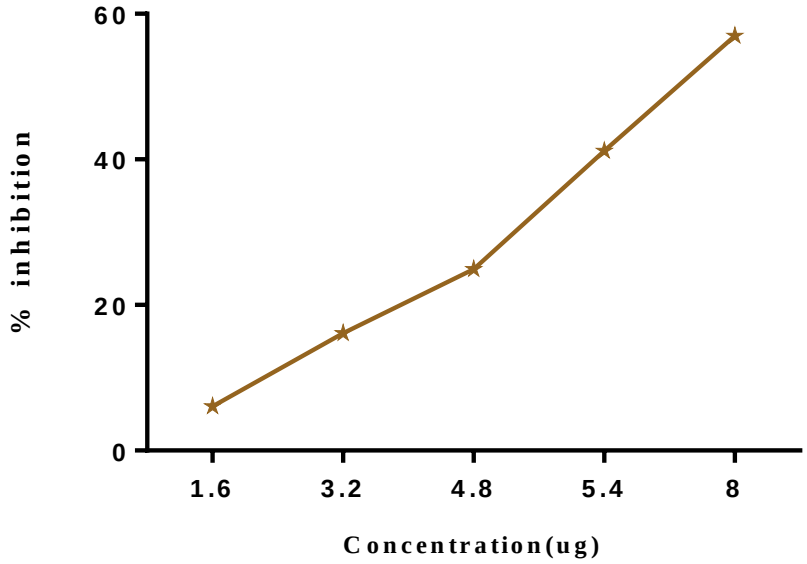


Figure 3.7. Effect of thiacloprid on AChE activity in IMR 32 cell line at different time intervals

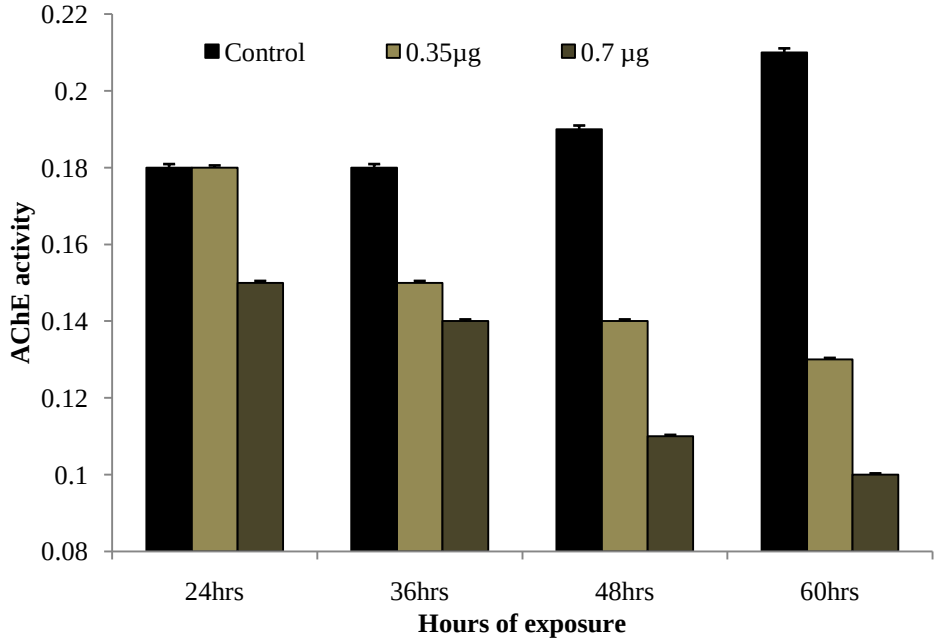


Figure 3.8. Effect of thiacloprid on NTE activity in IMR 32 cell line at different time intervals

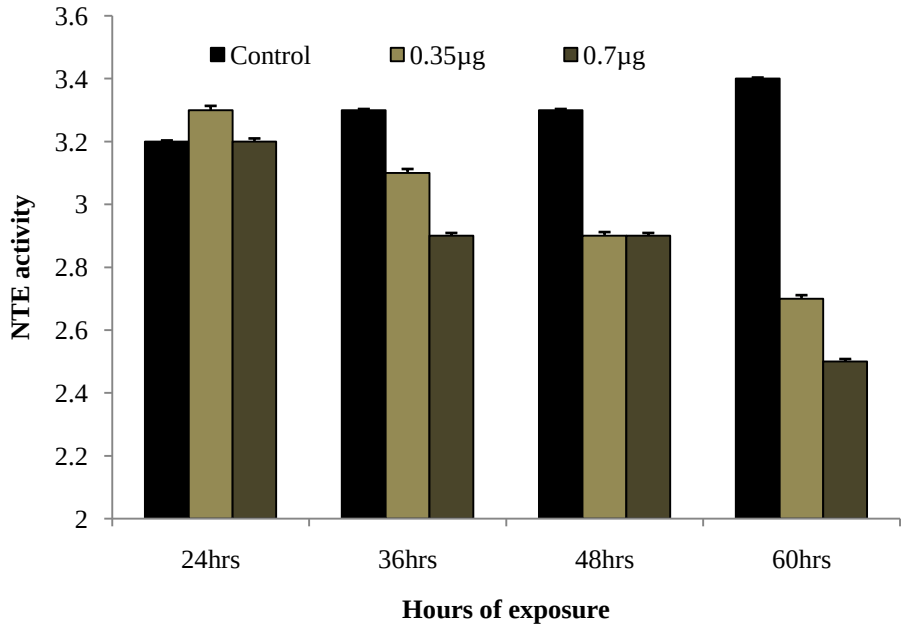
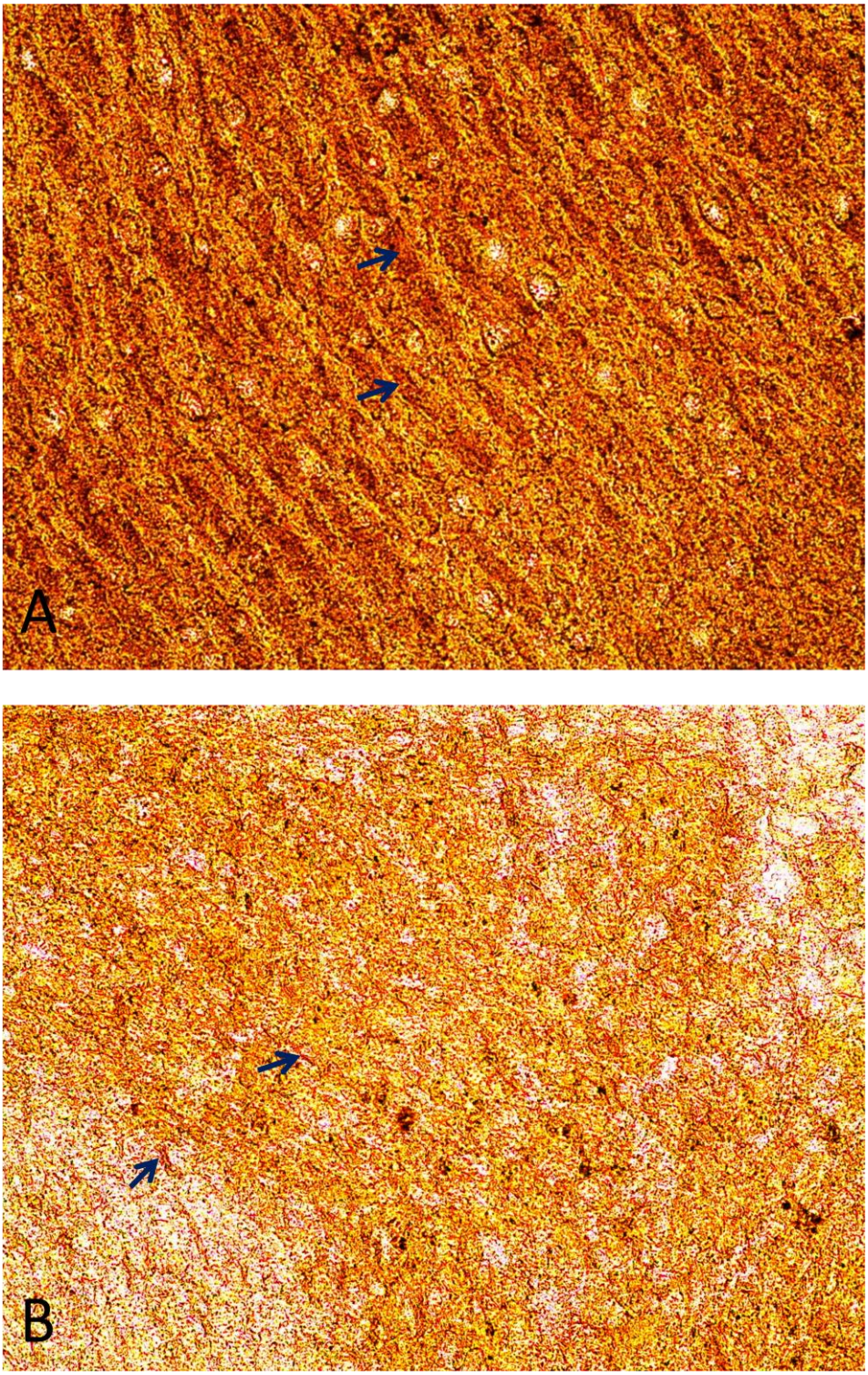
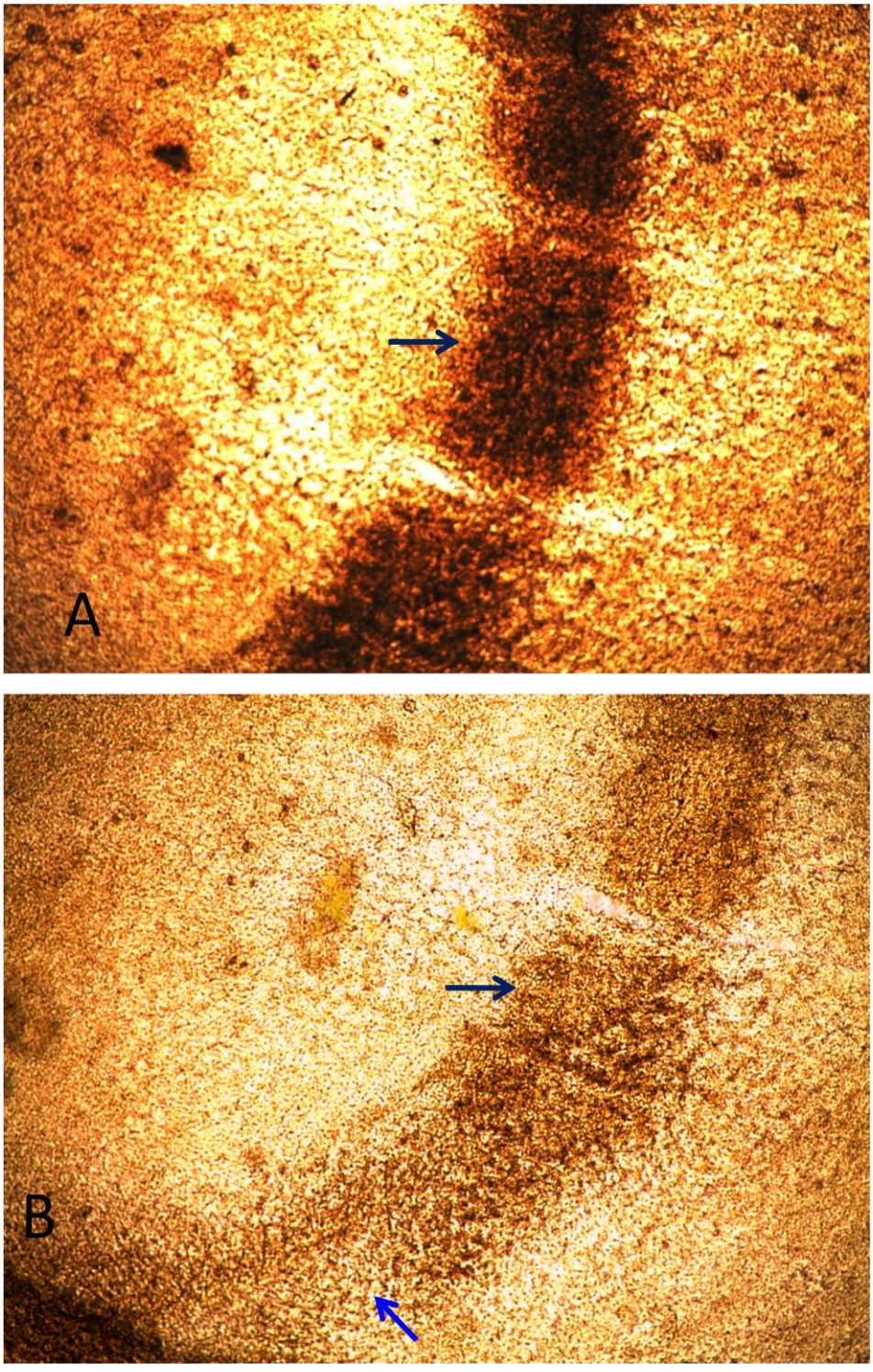


Figure 3.9 Histochemical localization of acetylcholinesterase(AChE) activity in the rat brain (40X).



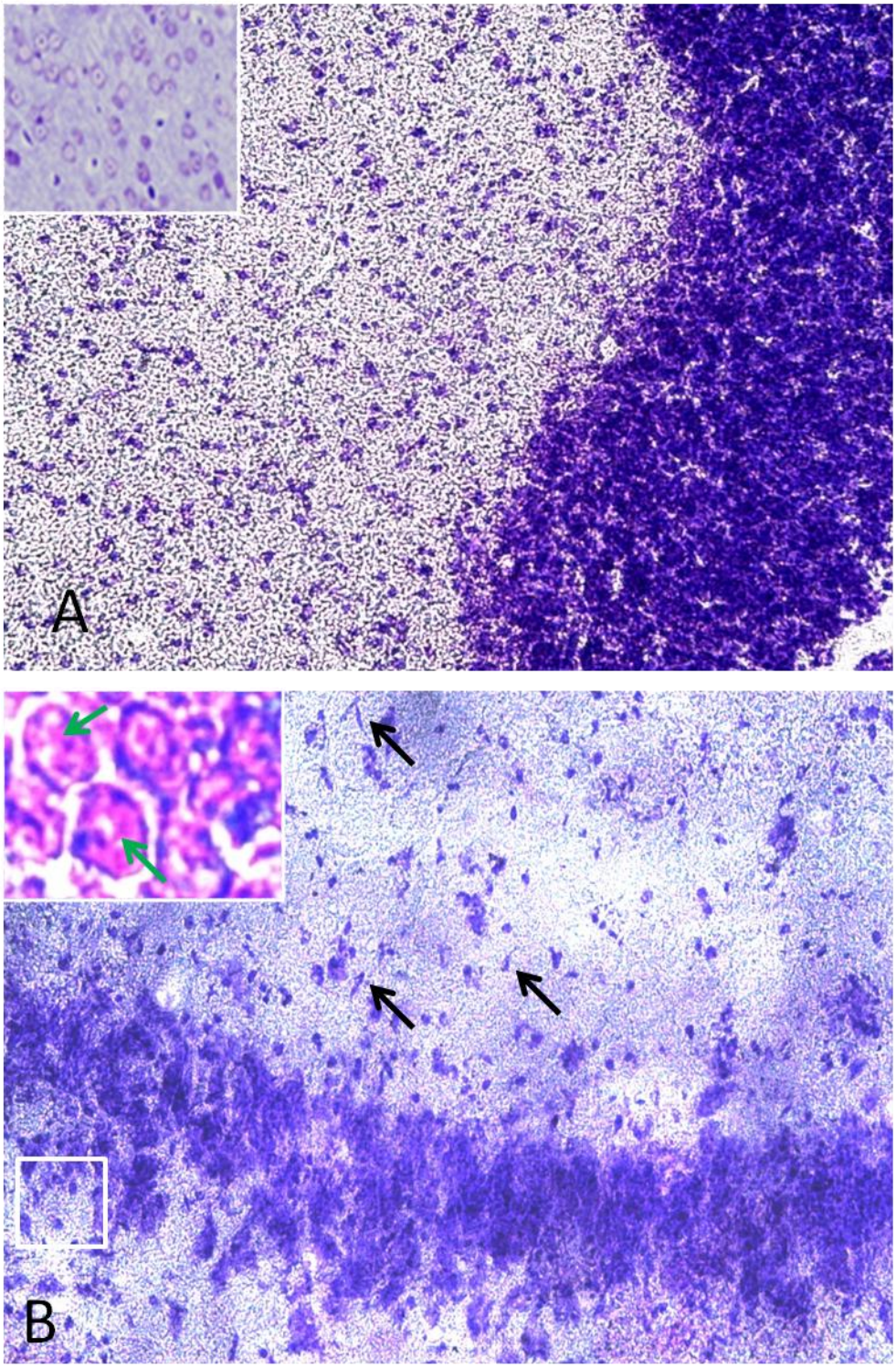
(A) AChE activities are localized in the brain of control rat (in brown color); (B) weak acetylcholinesterase activity was found in treated brain.

Figure 3.10 Histochemical localization of AChE activity in hippocampal area of rat brain (40X).



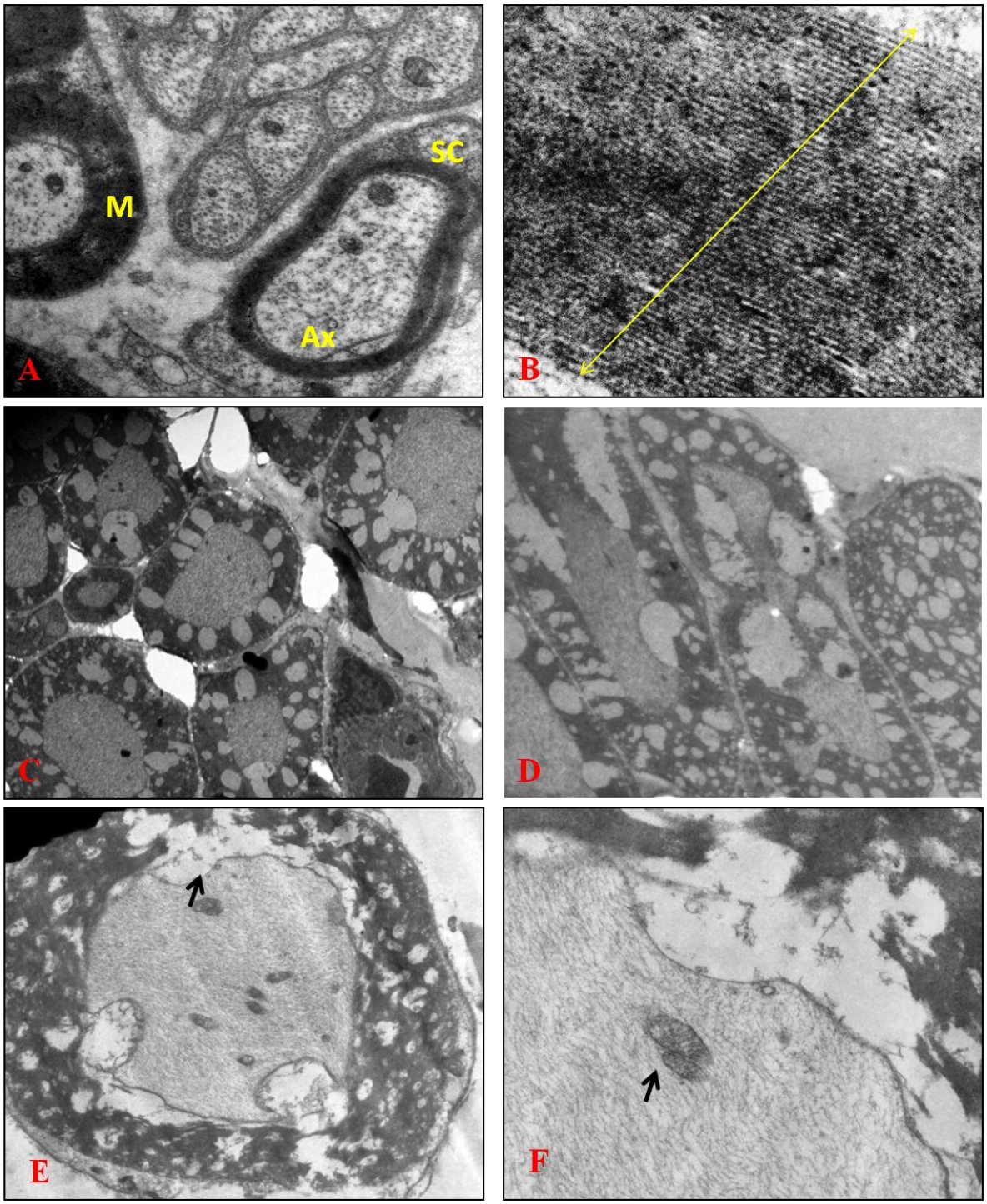
(A) Control hippocampal region AChE localized more due to its activity, (B) Treated section with less localization due to inhibition, deficiency of enzyme activity marked by blue arrow.

Figure 3.11 Nissl stained cerebrum of rat (40X).



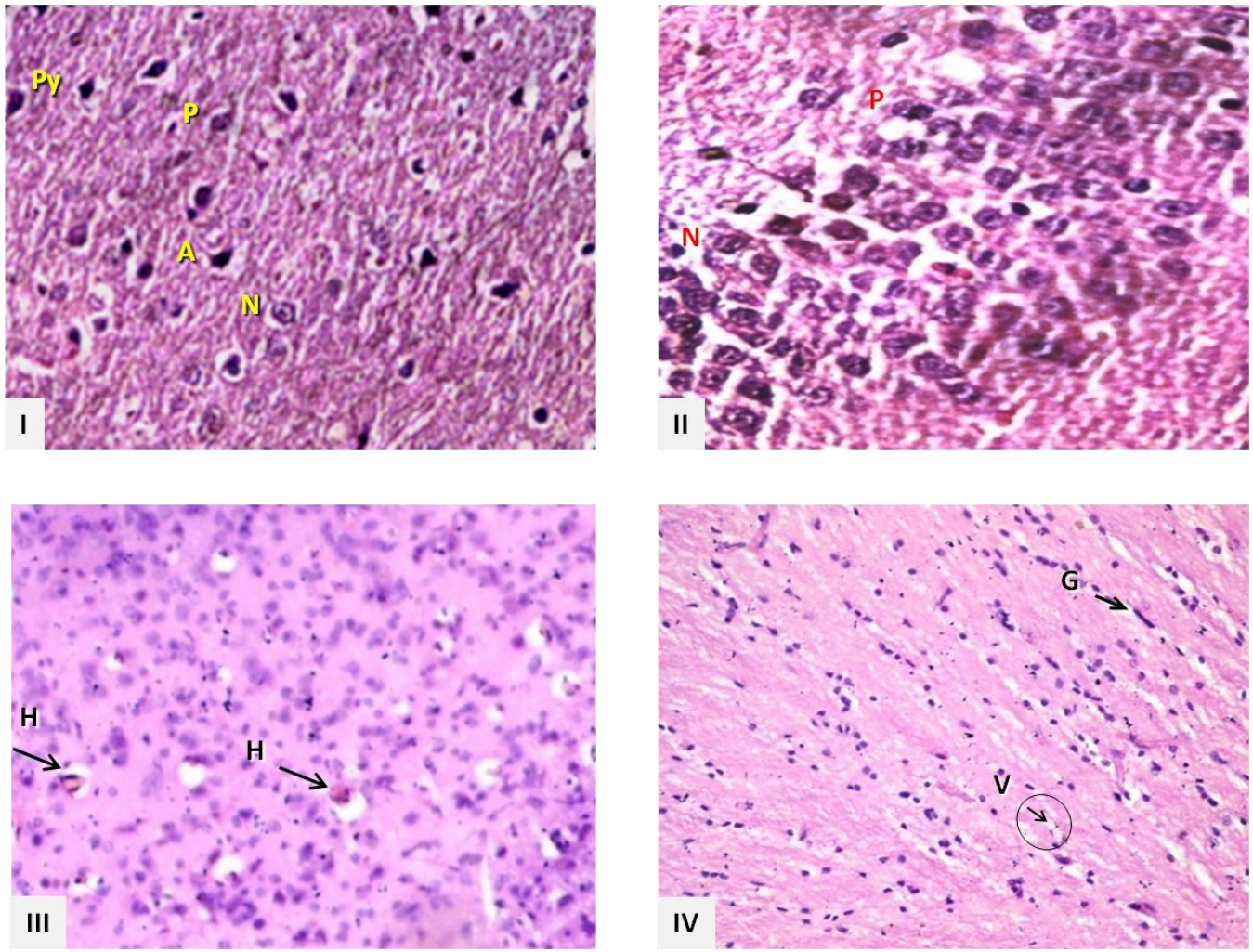
(A) control section with darkly stained Nissl body by cresyl violet, inset photograph of viable brain cell with Nissl body (B) Treated section showing necrotic cell (black arrow) and pyknotic cell (green arrow)at hippocampal region in inset photograph.

Figure 3.12 Transmission electron micrograph of nerve stained by lead uranyl acetate.



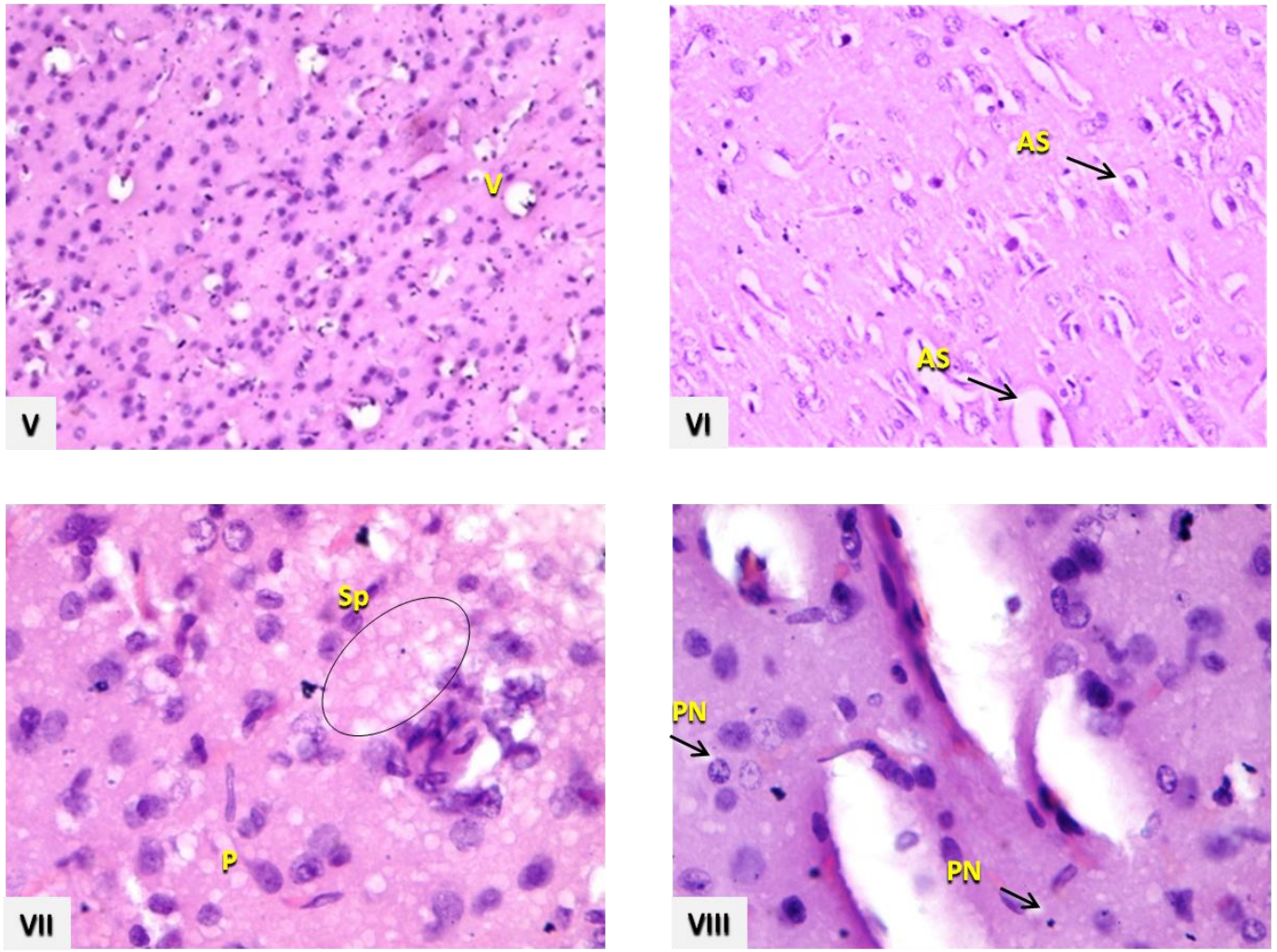
Control A(A and B): control animals, (C-F): thiacloprid treated animals. (A) Normal rats showing axon (Ax), myelin (M) and Schwann cell (Sc) (4000x). (B) Thickness and appearance of myelin in control rat (22000x). (C) Dysplasia of myelin sheath, disordered myelin layer structure, and vacuolar degeneration in thiacloprid treated rat (4000x) (D) Axon retraction (shrinking) and myelin degeneration (E) Detachment of myelin layers from axons (arrow), and severe loss of compact myelin. (E) Axonal separation from the myelin(8000x) (F) Crystallization of axoplasm, loss of mitochondrial membrane potential (arrow) (b) and complete separation from the myelin (14000X).

Figure.3.13 Light micrographs of the control and treated rat brain tissues.



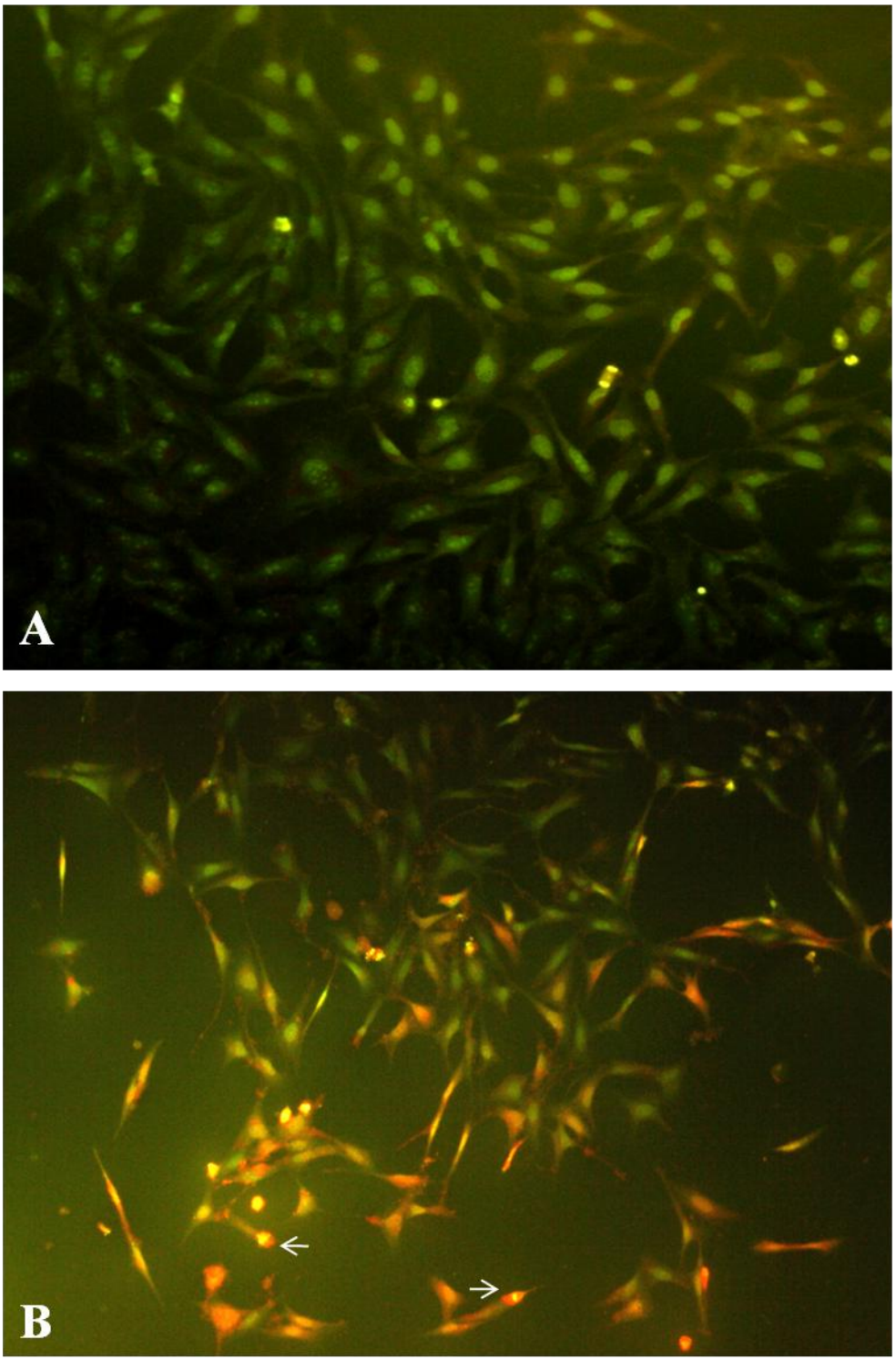
Control group (I and II). Thiacloprid treated group (III and IV). Tissue sections were stained with H&E. I: Control showing the various cells of cerebrum (A: Astrocytes, N: Neuron cell body, P: Purkinje cells, Py: Pyramidal cells) [40X]; II: Control showing the Thalamic region of brain [40X]; III: Treated rat brain showing minimal Haemorrhages [10X]; IV: Treated rat brain showing Gliosis (G) and Vacuolar degeneration (V) [10X].

Figure 3.14 Light micrographs of the brain tissue of Thiacloprid treated group.



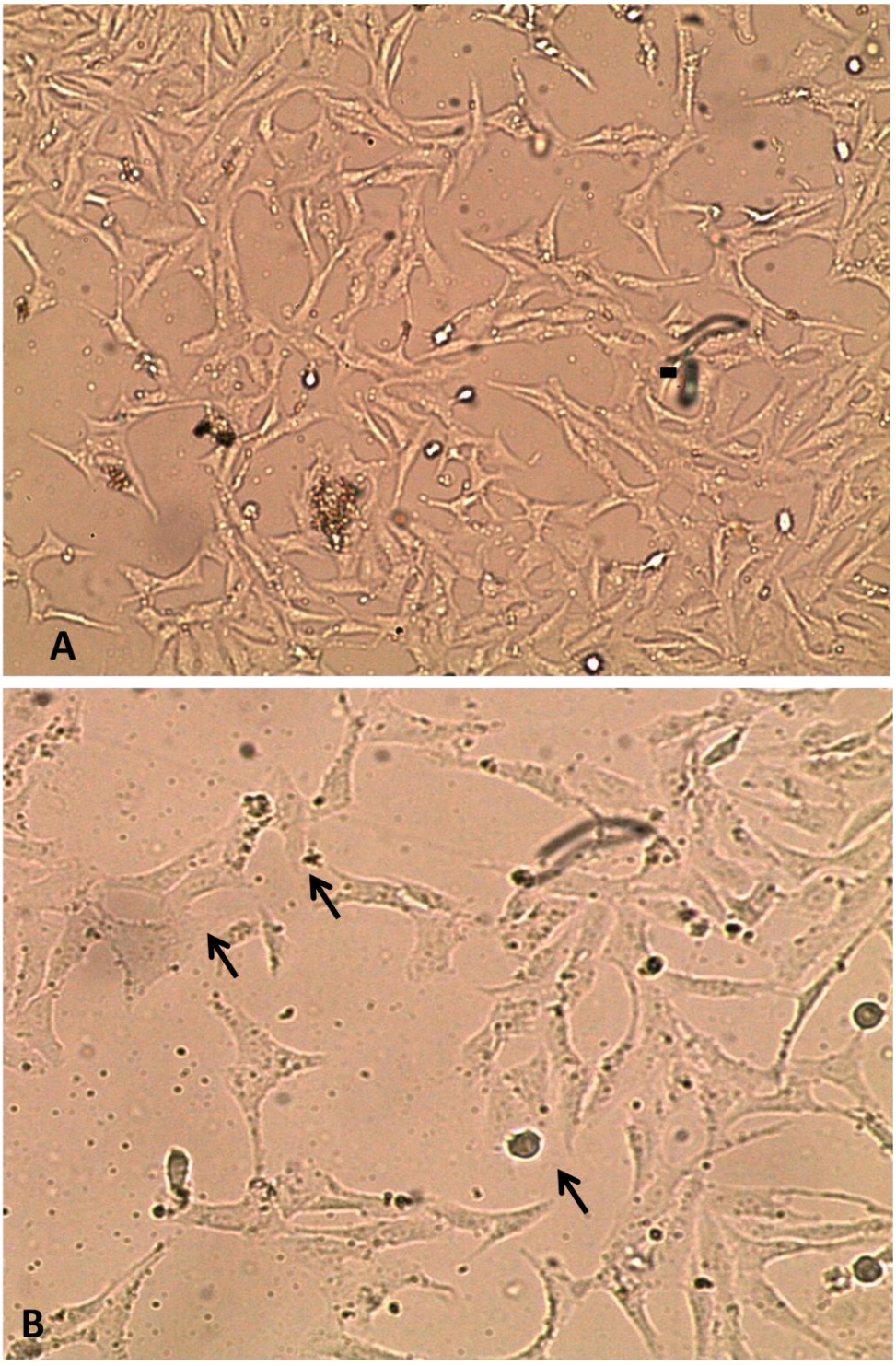
V to VIII (Thiacloprid treated group): Tissue sections were stained with H&E. V: Vacuolar degeneration (V) [20X]; VI: Axonal swelling (AS) [20X]; VII: Spongiform degeneration (SP) [100X]; VIII: Pyknotic nuclei (PN).

Figure 3.15 Florescent microscopy images of IMR 32 cells (AO/EB staining, 40X).



(A) Cell with green fluorescent is for viable cells of control; (B) Thiacloprid treated cells- Viable cells are with green fluorescent and nonviable are with red fluorescent (Blue arrow).

Figure 3.16 IMR 32 neuroblastoma cells (40X).



(A) Control cells: elongated neurite processes and healthy growth of cells; (B) Thiacloprid exposed cells with less neurite process elongation by inhibition of growth (black arrow).

CHAPTER 4

ASSESSMENT OF CYTOTOXIC AND GENOTOXIC POTENTIAL OF REPEATED DOSE OF THIACLOPRID IN RAT

INTRODUCTION

The introduction of new chemicals in nature may be responsible for numerous negative effects in humans, such as biochemical malfunctions or genetic instability (Tsutsui *et al*., 1984; Evans, 1985). Pesticides are widely used noxious chemicals in agriculture, either separately or in mixtures, and they invade the environment in large quantities (Demsia *et al*., 2007) and one of the major environmental health problems caused by these pesticides is their potential adverse effect on non-target organisms (Cavas *et al*., 2012).

Among currently available pesticides, neonicotinoids are considered to be the most important chemical class of insecticides introduced to the global market since the advent of synthetic pyrethroids (Jeschke and Nauen, 2008). Neonicotinoid insecticides are extensively used, both in crop protection and animal health applications (Chudoku, 2008; Cavas *et al*., 2012). They are currently registered in 120 countries and account for 25% of the world insecticide market (Jeschke *et al*., 2011). Thiacloprid is a member of the neonicotinoid group of insecticides, which act as selective agonist for the nicotinic acetylcholine receptors in insects. It is used against a wide range of insect pests in public health and in veterinary applications (Tomizawa and Casida, 2005).

Pesticides are very reactive compounds forming covalent bonds with various cellular biomolecules such as DNA and can consequently damage cells' genetic structure and/or interfere with metabolic processes. Thus, the genotoxicity of pesticides is currently a topic of worldwide concern (Cavas *et al*., 2012). It is also known that free radicals play an important role in the toxicity of pesticides and environmental chemicals (D'Almeida *et al*., 1997; Duzguner and Erdogan, 2012). Pesticide chemicals such as insecticides may induce oxidative stress leading to generation of free radicals and alterations in antioxidants or free radical scavenging enzyme systems (Kanbur *et al*., 2008; Duzguner and Erdogan, 2010). The data on experimental animals either *in vivo* or *in vitro* (John *et al*., 2001; Thapar *et al*., 2002; Singh *et al*., 2006) indicate that the enzymes associated with antioxidant defense mechanisms are altered under the influence of pesticides. Moreover, oxidative stress and DNA damage have

been proposed as mechanisms linking pesticide exposure to health effects such as cancer and neurological diseases. During metabolism of the insecticides, reactive oxygen species (ROS) and reactive nitrogen species (RNS) such as nitric oxide (NO) can be generated (Grisham *et al*., 1999; Duzguner and Erdogan, 2012).

Thiacloprid is the first among the cyano group of chloronicotinyl insecticides to be registered by Bayer AgroScience Ltd., with brand name of Calypso, Alanto, Biscaya, etc. (Elbert *et al*., 2001; Erdelen, 2001). Today, the use of this insecticide for controlling various species of pests on many crops is very common (Elbert *et al*., 2000). Because the use of thiacloprid has become increasingly widespread throughout the world, the assessment of its possible genotoxic and cytotoxic effects on living organisms is very important (Kocaman *et al*., 2012). EPA (2003) and FAO (2006) have even reported development of thyroid, ovarian, uterine and prostate cancer due to thiacloprid exposure in rats, suggesting it can be a potent carcinogen as well.

A literature survey showed that not many studies have been carried out on the potential genotoxic/cytotoxic (Zang *et al*., 2000; Feng *et al*., 2004, 2005; Karabay and Gunnetur, 2005; Demsia *et al*., 2007; Kocaman and Topaktas, 2007, Kocaman *et al*., 2012) and carcinogenic (Green *et al*., 2005; Green *et al*., 2005a; Pastoor *et al*., 2005) effects of some neonicotinoid insecticides. The available literature indicates that only one study has addressed the cytogenetic effect of thiacloprid *in vivo* and one another study has addressed the effect *in vitro*. Sekeroglu *et al*. (2011) reported that thiacloprid significantly decreased the mitotic index (MI) and binuclear cell numbers and significantly increased the chromosome aberrations (112.5mg/kg, for 24 h) and also caused a significant increase in micronucleated binuclear cells (22.5mg/kg/day, for 30 days) in rat bone marrow cells. Kocaman *et al*. (2012) reported that thiacloprid increased the chromosome aberration and sister chromatid exchange significantly at all tested concentrations of 75, 150, and 300µg/ml during an *in vitro* study on human peripheral lymphocytes.

However, the genotoxic as well as cytotoxic potential of thiacloprid have not yet been investigated in a single study. As observed in the previous chapters, thiacloprid exposure can induce oxidative stress, which may form DNA adducts and cause DNA damage. Increased DNA damage to bone marrow cells is known to lead to apoptosis or mutation in the cells. Apoptosis contributes to the pathogenesis of a number of diseases, including cancer (Thompson, 1995). Cell death in response to DNA damage, in most instances, has been shown to result from apoptosis (Thompson, 1995). Apoptosis is induced by many cytotoxic chemicals

and ionizing radiation and is characterized by morphological and biochemical changes such as chromatin condensation, nuclear fragmentation, formation of apoptotic bodies and DNA fragmentation at internucleosomal sites (Yusuke and Shosuke, 1996). Moreover, the DNA of apoptotic cells shows a unique fragmented pattern in gel electrophoresis experiments. Electrophoresis has proved to be a powerful molecular-level tool to distinguish between normal and apoptotic cells (Reed, 1994).

It is known that genotoxicity assessment of a particular compound is done by evaluation of its ability to induce any change in the chromosome structure and number such as gene mutations, chromosomal rearrangements, or deletions and loss or gain of a whole chromosome. Chromosome aberration (CA) and micronucleus (MN) formation are among the most widely used and well-established cytogenetic markers for determination of genotoxicity of any compound (Carrano and Natarajan, 1988). The structural CA formation results from DNA-level damage that is associated with an increased risk of cancer and can also be used to predict genotoxic risk of potentially mutagenic and carcinogenic chemicals (Bonassi *et al*., 2004; Norppa *et al*., 2006; Kocaman *et al*., 2012).

The rodent bone-marrow MN test is the most widely used short-term *in vivo* assay for the identification of genotoxic effects such as chromosome damage and aneuploidy associated with mutagenesis and carcinogenesis (Heddle, 1973; Schmid, 1973; Vanderkerken *et al*., 1989; Demsia *et al*., 2007). MN can be formed from acentric chromosomal/chromatid fragments or from whole chromosomes/chromatids that fail to be segregated to daughter nuclei during mitotic cellular division, because they did not attach properly with the spindle during the segregation process in anaphase (Fenech, 2007; Bonassi *et al.,* 2004; Fenech *et al*., 2011). Thus, both clastogenic and aneugenic effects (leading to structural and numerical CAs, respectively) can be determined with the micronucleus assay (Norppa and Falck, 2003; Kocaman *et al*., 2012).

Genotoxic effects of different types of neonicotinoid insecticides have been investigated by several researchers (Karabay and Gunnehir, 2005; Kocaman and Topaktas, 2007; Costa *et al*., 2009; Stivaktakis *et al*., 2010; Cavas *et al*., 2012). However, a literature survey showed that the studies dealing with the genotoxic and cytotoxic effects of thiacloprid are extremely rare (Sekeroglu *et al*., 2011; Kocaman *et al*., 2012). As thiacloprid is the most frequently used neonicotinoid pesticide, it is possible that its exposure may have potential toxic effects on non-target organisms such as mammals, a notion which remains to be properly tetsed. Efforts in this direction have already revealed the hepatotoxic, thyrotoxic and neurotoxic effects of

thiacloprid in the mammalian system (Chapters 1, 2 and 3). Hence, the current study was designed to investigate the potential genotoxic and cytotoxic effects of thiacloprid on mammalian bone marrow, using SD rat as the animal model. To fulfill this objective, various ROS parameters were estimated to examine the oxidative stress generated by repeated thiacloprid exposure in rat bone marrow. Potential DNA damage induced by thiacloprid was assessed by MN test, chromosomal aberration test and comet assay in rat bone marrow. Possible cell death induced by thiacloprid exposure was also assessed by acridine orange and ethidium bromide staining of bone marrow smears and results were confirmed by DNA ladder assay.

MATERIAL AND METHODS

Male Sprague Dawley (SD) rats (weighing 225–275gm) were obtained from CPCSEA approved animal breeders and housed in well-ventilated cages in a temperature/humidity-controlled room, with access to water and food *ad libitum*. All animal experiments were approved by IAEC as per the standard guidelines of CPCSEA, India. Thiacloprid (Alanto 240, 21.7%SC) was purchased from the local market in Vadodara. Test compound was dissolved in distilled water to obtain the desired dose concentrations of 50mg/kg body weight and 100mg/kg body weight (1/30 and 1/15 of its LD50 value respectively). Rats were given oral doses of thiacloprid by intubation once in a day for 28 days and 90 days (subacute and subchronic studies respectively). For both the studies, animals were divided into three groups as control, low dose treatment group [50mg/kg body weight (LTD)] and high dose treatment group [100mg/kg body weight (HTD)] with 5 animals in each group.

The rats were euthanized under mild diethyl ether anaesthesia after overnight fasting. Bone marrow was removed from the femur bones in RPMI 1640 medium and then centrifuged at 3000rpm for 5 minutes. The pellet was resuspended again in RPMI 1640 medium. Bone marrow smear was prepared on clean glass slide and remaining marrow was used for evaluating ROS parameters.

Protocol I: Oxidative stress parameters

The lipid peroxidation product, malondialdehyde (MDA), was estimated by thiobarbituric acid (TBA) method (Janero, 1998) and intensity of colour was measured at 532nm. Catalase activity was measured at 590nm by the method of Sinha *et al*. (1972). Marklund and Marklund method (1974) was adopted to estimate the superoxide dismutase (SOD) activity at 420nm.

The activity of glutathione peroxidase (GPx) was determined by the method of Rotruck *et al*. (1973) and the GSH content remaining after the reaction was measured by the method of Ellman *et al*. (1961) at 412nm. The method described by Habig *et al*. (1994) was used for estimating the activity of Glutathione-S-transferase (GST) at 340nm. The reduced glutathione level was measured at 412nm by the method of Beutler *et al*. (1963).

Protocol II: Cytotoxicity and Genotoxicity parameters

Bone marrow smear was fixed, followed by staining with acridine orange:ethidium bromide (AO/EB) (1:1) solution for 2 minutes and then washed with PBS. At least 1000 bone marrow cells were examined for each group under fluorescent microscope (Leica DM2500) using a fluorescein filter and 40X objective. Green fluorescence is observed for viable cells and red fluorescence for necrotic cells, while the apoptotic cells show red and green fluorescence with apoptotic characteristics.

Micronucleus (MN) test was performed according to prescribed protocol of Romagna and Staniforth (1989). Bone marrow smear was stained for 5 minutes with 10µg/ml acridine orange fluorescent dye followed by washing with PBS twice. Slides were observed under fluorescent microscope to score micronuclei. 1000 cells were counted per rat. Bone marrow smear was stained with 2% Geimsa stain to score polychromatic erythrocyte (PCE) (immature or intermediate erythrocyte with ribosome) and normochromatic erythrocyte (NCE) (mature erythrocyte without ribosome). PCE/NCE ratio was calculated for analysis of cytotoxicity of test chemical. Micronucleated PCE (MNPCE) and micronucleated NCE (MNNCE) were also scored with Geimsa staining.

Evans *et al*. (1964) method was used for metaphase chromosome preparation. Rats were weighed and colchicine (4mg/kg body weight) was administered intraperitoneally 2.5 hrs prior to the harvest of bone marrow cells. The collected bone marrow cells were agitated in 0.6% KCl and then incubated at 50ºC in water bath for 45 minutes, followed by centrifugation. The pellet was fixed using 3:1 (Methanol: Glacial acetic acid) fixative, agitated properly and then centrifuged at 3000 rpm for 10 minutes to again collect the pellet. This step was repeated twice. Cells were spread on a chilled glass slide which was then kept on a hot plate at 60ºC for 10 seconds. Finally slides were stained with 2% Geimsa for 10 minutes.

Comet assay was performed in rat bone marrow cells, according to the *in vivo* comet assay guidelines of Tice *et al*. (2000), as described by Saquib *et al*. (2009). Bone marrow cells were mixed with 1% Low Melting Agarose (LMA), then layered onto slides pre-coated with 1%

agarose and kept at 4°C for 10 min. After gelling, another layer of LMA was added onto it. Cells were lysed overnight in a lysis buffer and then rinsed in PBS twice before subjecting to DNA denaturation in cold electrophoretic buffer at 4°C for 20 minutes. Electrophoresis was performed at 25V (300mA) at 4 °C for 30 minutes. Slides were then washed with neutralization buffer thrice. Each slide was stained with 20μg/ml ethidium bromide solution and analyzed at 40X magnification (excitation wavelength of 515-560nm and emission wavelength of 590nm) using a fluorescence microscope (Leica DM2500). Images from 100 cells (50 from each replicate slide) were randomly selected and subjected to image analysis with CometScore software (TriTek Corporation, Virginia). Mean values of the Olive tail movement (OTM), tail length (μm) and % DNA in tail were separately analyzed for statistical significance. To quantify the DNA damage, tail length (TL) and tail movement (TM) were evaluated.

Statistical Analysis

The data are expressed as the Mean ± standard error. Differences between the groups were assessed by one way ANOVA using the SPSS software (version 12.0). The comparisons between the groups were made using a post hoc Bonferroni test. Differences were considered significant at $p \leq 0.05$.

RESULTS

In Table 4.1, bone marrow MDA and GSH levels have been given. Bone marrow MDA levels observed for animals of HTD group in the subacute study and also for both LTD and HTD groups in subchronic study were significantly increased ($p \leq 0.05$) in comparison with the corresponding control groups. Furthermore, the increase in TBARS level was also observed for the bone marrow of animals in the LTD group of the subacute study; however the difference was not significant (Figure 4.1). A pronounced decrease in treated rat bone marrow GSH level as compared to control GSH was noted during both the treatment periods. This decrease was statistically significant for LTD group ($p \leq 0.05$) in the subacute study and both LTD ($p \leq 0.05$) and HTD ($p \leq 0.01$) groups in the subchronic study. GSH value observed for the LTD group was also less than the control group, but the difference was not statistically significant. (Table 4.1, Figures 4.2 and 4.3).

Table 4.2 shows the values for antioxidant enzyme activity in the bone marrow of the experimental rats given subacute exposure to thiacloprid. Activity of catalase was observed to be decreased in the both the treatment groups as compared to control group. GST activity was

also found to be decreased in treated rats compared to control animals with significantly lower activity observed in HTD group ($p \leq 0.05$). No significant decrease in GPx activity was observed for the treatment groups as compared to the control group in the subacute study (Figure 4.2).

Effect on antioxidant enzyme activity after 90 days of thiacloprid administration is shown in Table 4.3. Activity of catalase enzyme was found to be decreased in treated rats as compared to control rats, with the difference being significant for the HTD group ($p \leq 0.05$). GST and GPx activities in bone marrow of treated rats of both the dosage groups were observed to be significantly decreased as compared to reference group of rats ($p \leq 0.05$) (Figure 4.3). However, it was noted that SOD activity in the bone marrow was not affected by thiacloprid exposure during both the study periods (Tables 4.2, 4.3; Figures 4.2, 4.3).

Data showing the effect of thiacloprid on rat bone marrow cells giving rise to micronuclei is given in Table 4.4. Thiacloprid induced a significant increase ($p \leq 0.05$) in percent frequency of MNPCE number in both the treatment groups in the subacute study. Even repeated 90 days exposure to test compound induced a significant increase in frequency of MNPCE in rat bone marrow of both the treatment groups ($p \leq 0.05$). An increase in percent frequency of MNNCE in treatment groups as compared to control group was also observed in the subacute study, but this result was not statistically significant. However, the increase in MNNCE frequency observed for the HTD group as compared to control group in the subchronic study was significant ($p \leq 0.05$) (Figure 4.4). Total percent frequency of micronucleus induced by thiacloprid was observed to be significantly higher ($p \leq 0.05$) in both the treatment groups as compared to the control group in subacute study. Same trend was observed for the LTD ($p \leq 0.05$) and HTD ($p \leq 0.01$) groups in the subchronic study as well (Figure 4.4) Figure 4.8 shows bone marrow cells with a micronucleus in the thiacloprid treated rats.

The cytotoxic potential of thiacloprid was evaluated through the ratio of PCE to NCE among total cell count of bone marrow. The calculated PCE/NCE ratio is given in Table 4.4. The ratio of PCE/NCE was observed insignificantly decreased for both the treatment groups in the 28 days study, but a significantly decreased ratio of PCE/NCE was observed for both the treatment groups as compared to control group in the subchronic study ($p \leq 0.05$) (Figure 4.5).

Table 4.5 summarizes the effects of thiacloprid on metaphase chromosomes of rat bone marrow cells following 28 and 90 days exposure. Thiacloprid induced structural as well as numerical aberrations in rat bone marrow cells as encountered in the present investigation.

Among these, special emphasis has been put on gaps, breaks, translocation, fragmentation, centric association (dicentric and ring chromosome) and polyploidy. The increased frequency of chromatid aberrations with reference to gaps and breaks was observed to be highly significant for HTD group in both subchronic ($p \leq 0.01$) as well as subacute studies ($p \leq 0.01$) as compared to control values. Frequency of these aberrations was also significantly higher for the LTD group ($p \leq 0.05$) in the subchronic study. Thiacloprid treatment also significantly increased the frequency of chromosome type aberrations in both the treatment groups in the subacute ($p \leq 0.05$) as well as the subchronic study ($p \leq 0.001$). Chromosomal aberrations in the treatment groups of the subchronic study were observed to be increased almost two fold as compared to control group (Figure 4.10). Among chromosomal aberrations, fragmentation of chromosome was the most frequently encountered aberration (Figure 4.10 C & D). Frequency of ring chromosome was observed insignificantly increased due to thiacloprid exposure (Figure 4.10). Translocation and polyploidy were the least encountered aberrations in comparison to other chromosomal aberrations. Figure 4.10 represents the various types of chromosomal aberrations induced by thiacloprid in rat bone marrow cells.

Genotoxicity of thiacloprid on rat bone marrow cells was assessed using comet assay to analyze the extent of DNA damage at the level of individual cells. Results generated by CometScore software are illustrated in Table 4.6. Single cell DNA damage as assessed by comet tail length was found to be highly significant in HTD group of rats as compared to control group for both the studies ($p \leq 0.01$). Rats exposed to thiacloprid at low dose in the subchronic study also showed significantly increased length of comet tail ($p \leq 0.05$). A highly significant olive tail movement of comet was observed for the HTD groups ($p \leq 0.01$) as compared to control group in both the studies. Tail movement in the LTD group was also significantly higher ($p \leq 0.05$) than the control group in the subacute study. % Tail DNA of comet was also observed to be significantly higher than the control group, in HTD groups of both the studies as well as in the LTD group of the subchronic study ($p \leq 0.01$) (Figures 4.6 a & b). DNA damage induced by thiacloprid as assessed by comet assay is shown in Figure 4.11.

Thiacloprid-induced cell-death, as observed by DNA gel electrophoresis, is presented in Figure 4.12. DNA fragmentation was clearly evident in treated rat bone marrow (L-1 and H-1 represent LTD and HTD groups of subacute study; L-2 and H-2 represent LTD and HTD groups of subacute study), whereas such fragmentation was absent in control (C-1 for subchronic and C-2 for subacute) rat bone marrow. Moreover, nuclear condensation, cell shrinkage and fragmentation into apoptotic bodies, which are characteristics of apoptosis were

observed in the thiacloprid treatment groups as visualized in AO/EB stained bone marrow smears (Figure 4.9). Acridine orange intercalates with DNA and RNA, making the former appear green while the latter stains red. Thus a viable cell has bright green chromatin in its nucleus and red-orange cytoplasm. Ethidium bromide is only taken up by nonviable cells. Ethidium bromide intercalates into DNA, making it appear orange, but binds only weakly to RNA, which may appear slightly red. Thus a dead cell has bright orange chromatin (the ethidium overwhelms the acridine) and its cytoplasm, if it has any contents remaining, appears dark red. Cells that have undergone necrosis have the fluorescent features of nonviable cells but do not have apoptotic nuclear morphology (Ribble *et al*., 2005). Based on these features, it was observed that a greater number of cells were in the apoptotic phase in thiacloprid treated groups and also that thiacloprid induced cell apoptosis in a dose dependent manner (Figure 4.9).

DISCUSSION

The present study was designed to evaluate the cytotoxic and genotoxic effects of the neonicotinoid thiacloprid. In this regard, several studies were carried out and one among these was the evaluation of oxidative defense mechanisms in the bone marrow of thiacloprid treated rats. Results obtained demonstrated that exposure of rats to thiacloprid decreased the activity of antioxidant enzymes as well as the GSH level and increased the TBARS level, indicating that that the test compound may result in the impairment of antioxidant mechanisms and metabolic detoxification in organs. As such, the decrease in antioxidant enzyme levels is interpreted as an indirect inhibition of their activity by their binding with oxidative molecules produced during pesticide metabolism after exposure (Duzguner and Erdogan, 2012).

Neonicotinoid exposure can promote lipid peroxidation and hence, the LPO activity was also assessed during the current thiacloprid induced genotoxicity study. Lipid peroxidation results in altered membrane function and production of toxic and reactive aldehydes, mainly MDA, which is capable of interacting with proteins or DNA and thereby possibly promoting mutagenesis (Cheeseman, 1993; Toyokuni, 1996). Oxygen free radicals generated due to exposure to pesticides can cause tissue damage by triggering several oxidative mechanisms and lipid peroxidation (Kanbur *et al*., 2008). Indeed, Duzguner and Erdogan (2012) reported that neonicotinoid imidacloprid leads to lipid peroxidation in various organs of rats. Similar result of the significant increase of MDA, the index of lipid peroxidation, in bone marrow

following thiacloprid treatment is an important evidence of oxidative stress in the present study.

Along with an increase in lipid peroxidation, activities of several antioxidant enzymes such as catalase, GST, GPx and SOD was observed to be significantly decreased in the plasma of rats administered with thiacloprid in the current study. The relationship of pesticide exposure to a decrease in antioxidant enzyme (SOD, catalase, and GPx) levels in the erythrocytes of humans who had been long-term exposed to pesticides has been reported by Lopez *et al*. (2007). Thiacloprid being a very new insecticide, oxidative stress induced by this compound is not reported in the literature. However, results obtained in the current study are similar to those reported by Duzguner and Erdogan (2012). They reported that activities of SOD, catalase and GPx were found to be decreased in the bone marrow of rats after oral administration of imidacloprid.

GSH is an important intracellular antioxidant (hydrogen-donating compound) that spontaneously neutralizes several electrophiles and reactive oxygen species (Lu, 1999; Kent *et al*., 2003); and it also plays a key role in maintaining the redox status of the cell (Rana *et al*., 2002). The present study indicated that subacute and subchronic treatment of thiacloprid also decreased GSH content in the bone marrow of rats, which makes cells more susceptible to oxidative damage, particularly during increased free radical production. Present results are comparable with those of Duzguner and Erdogan (2012), who demonstrated that acute and subacute treatment with the neonicotinoid imidacloprid reduced the glutathione level in the bone marrow of rats.

Elevated level of ROS induces oxidative stress which leads to oxidative DNA damage and micronucleus formation, a probable mechanism of genotoxicity (Ritesh *et al*., 2011). Higher expectation of DNA damage is associated with higher frequency of micronucleus in cells and so micronucleus assay is widely used to assess the genotoxic potency of compounds. Furthermore, it also gives indication concerning the cytotoxic potential of the tested compound (Fenech, 2008). *In vivo* micronucleus assay (MN) is most often performed without cytokinesis-block, and micronuclei in immature (polychromatic) erythrocytes are counted in the bone marrow and/or peripheral blood cells of animals, usually rodents (Hayashi *et al*., 1994; Hamada *et al*., 2001; Sekeroglu *et al*., 2012). In the present study as well, this assay was used to find the genotoxic potential of thiacloprid. Findings of the study indicated that thiacloprid induced significant increase in percent frequency of micronucleus in rat bone marrow cells as compared to the control group. Sekeroglu *et al*. (2011) reported that a commercial formulation

of thiacloprid significantly increased the frequency of micronucleus (22.5mg/kg/day, for 30 days) in rat bone marrow cells. Micronucleus frequency was also observed to be significantly higher in human peripheral lymphocytes exposed to thiacloprid (300µg/ml, 48 hrs treatment period) in an *in vitro* study done by Kocaman *et al*. (2012). In addition, results of current study also find support from previous findings obtained by researchers (Kocaman and Topaktas, 2007; Cavas *et al*., 2012) for a commercial formulation of acetamiprid (like thiacloprid, acetamiprid belongs to the chemical subclass of neonicotinoids known as cyanoamidine), which significantly induced MN formation.

An increased number of polychromatic erythrocytes with micronucleus were observed in thiacloprid-exposed rats as compared to the control group of rats. It is considered that a decrease in the ratio of polychromatic erythrocytes (PCE) to normochromatic erythrocytes (NCE) (P/N) in the micronucleus test is an indicator of bone marrow toxicity induced by mutagens. However, the exact meaning of fluctuation in the P/N ratio is not yet known (Suzuki *et al*., 1989). Decreased P/N ratio is suggestive of the impairment of the erythropoietic system of bone marrow which resulted into more number of denucleated NCE in bone marrow instead of these entering into peripheral blood stream. This impairment can be ably supported by the decrease in total count of NCE blood caused due to thiacloprid exposure observed during the course of the study. Blood haemogram of treated rats revealed a low RBC count in treated rat blood (Heamogram data not included in thesis). The P/N ratio is also said to be an important parameter to monitor progression/regression of cancer that is capable of affecting erythropoiesis in bone marrow (Gerashchenko *et al*., 2012).

A significant increase in the number of both, chromatid and chromosomal type aberrations found in rat bone marrow after the subacute and subchronic exposure to thiacloprid in the present study suggests that this pesticide could have potential clastogenic effects. This result also finds support from other studies conducted with thiacloprid, which mention a significant increase in chromosome aberrations in rat bone marrow and lymphocyte culture (Sekeroglu *et al*., 2011; Kocaman *et al*., 2012). Although xenobiotic agents mostly lead to the appearance of chromatid aberrations, there is evidence that due to their structure and metabolites, some of them could also induce chromosome type of aberrations (Kocaman *et al*., 2012), as shown in the present study. The chromosome type of aberrations could also arise due to misrepair of lesions in the G_0 stage of circulating lymphocytes as well as derived aberrations from precursor cells in bone marrow and thymus, as suggested by Carrano and Natarajan (1988).

Results of the chromosomal aberration analysis obtained in the current study indicate the possibility that subacute and subchronic exposure to thiacloprid could induce a significant increase in the level of DNA damage. The comet assay is the most rapid and sensitive method to evaluate DNA damage induced by genotoxic agents both *in vitro* and *in vivo* (Tice *et al*., 2000). The thiacloprid doses used in the present study induced considerable DNA damage, as could be observed from the significant increase in comet tail length and olive tail movement in comet assay of bone marrow cells of treated rats as compared to the control. Calderon-Segura *et al*. (2012) observed similar finding in comet assay with significantly increased comet tail length with human peripheral blood culture exposed to Calypso (a formulative product of thiacloprid, 480 SC). Our results are also in agreement with data obtained from *in vitro* genotoxic studies performed with the neonicotinoid insecticide imidacloprid. Exposure to 1mM imidacloprid that was metabolically activated *in vitro* with a rat liver S9 mixture was reported to produce calf thymus DNA adducts (Shah *et al*., 1997).

Programmed cell death selectively removes the most heavily damaged cells from a population (Reed, 1994; Thompson, 1995). Overproduction of ROS damages the cell and leads to apoptosis, so that unhealthy cells with unrepaired DNA are cleared from the normal cell population. Results showing the DNA ladder pattern on the agarose gel for the treated groups during electrophoretic study clearly revealed the apoptotic bone marrow cell death being enhanced by thiacloprid exposure. Similar reports regarding neonicotinoid exposure are still at large. However, on comparing the cell death induced by the neonicotinoid thiacloprid with toxic effects of nicotine, the results of the current study seem to have supportive evidence, because nicotine is a known carcinogen and is reported to induce cancer by increasing apoptotic cell death (Roy *et al*., 1998). Also cell apoptosis seen in brain sections of thiacloprid treated groups in the neurotoxicity study (Chapter 3) further support its potential of inducing apoptosis.

Rat bone marrow smears were stained with AO-EB and results of this study also point towards more pronounced apoptotic cell death in the bone marrow of treated rats. The occurrence of apoptotic cells was more prominently seen in bone marrow of rats given subchronic exposure as compared to those given subacute exposure to thiacloprid. There were significant morphological changes observed in bone marrow cells after exposure to thiacloprid. These included cell shrinkage, chromatin agglutination, marginalization, nuclear fragmentation, and apoptotic body formation. During literature survey one came across only a single *in vitro* study, which reported that thiacloprid significantly reduced cell viability (Calderon-Segura *et*

al., 2012) but whether or not it could induce cell death was not worked out. In addition to this report, Bar *et al*. (2012a, 2012b) reported induction of apoptosis in reproductive organs of rats exposed to imidacloprid and clothianidin. Apart from these reports, comparable studies relating neonicotinoid exposure and induction of apoptosis are exceptionally rare.

CONCLUSION

The current study was endeavoured to explore possible genotoxic and cytotoxic mechanisms of the neonicotinoid insecticide thiacloprid on the non-target mammalian system. Results show that thiacloprid genotoxicity was reflected as development of differential DNA damage and its effects on cellular viability. Thiacloprid exposure induced cytotoxic and genotoxic effects in rat bone marrow cells, and its toxicity may be mediated through ROS as evidenced by a significant production of MDA, an end product of lipid peroxidation and reduced activity of antioxidant enzymes. These observations suggest that oxidative stress plays an important role in thiacloprid-induced cytotoxicity and genotoxicity in mammalian cells. Thiacloprid was also found to cause DNA damage in bone marrow cells and induce apoptotic cell death, providing clear evidence that it may be mitogenic. Moreover, increased frequency of micronuclei and chromosomal aberrations induced by thiacloprid, undoubtedly proves its potential as a prominent genotoxicant in mammals.

Table 4.1. Malondialdehyde (MDA) and glutathione (GSH) levels in bone marrow of control and thiacloprid treated rats

Group	MDA (nmol/min/mg tissue)		GSH(µg/ gm tissue)	
	Subacute	Subchronic	Subacute	Subchronic
Control	4.9±0.26[@]	5.1±0.15	5.7±0.13	6.7±0.13
Low dose (50mg/kg b.w.)	5.1±0.24	5.9±0.21*	5.3±0.11	6.1±0.14*
High dose (100 mg/kg b.w.)	6.0±0.29*	6.1±0.23*	5.2±0.14*	5.8±0.12**

Table 4.2. Effect of subacute thiacloprid exposure on antioxidant enzyme activity in bone marrow of rats

Group	Control	Low dose (50mg/kg b.w.)	High dose (100mg/kg b.w.)
SOD (% inhibition /min/mg tissue)	3.6±0.11[@]	3.76±0.13	3.98±0.23
Catalase (µmole H_2O_2 liberate/ minute/ mg protein	7.8±0.15	7.4±0.13	7.6±0.15
GST (µmoles of GSH / minute/ mg protein)	12.1±0.2	11.7±0.17	11.3±0.21*
GPx (mM of GSH consumed/ mg tissue	13.5±0.25	13.4±0.19	13.1±0.24

[@]Data expressed as Mean±SEM; n=5 for each group; *p≤0.05 and **p≤0.01

Table 4.3. Effect of subchronic thiacloprid exposure on antioxidant enzyme activity in bone marrow of rats

Group	Control	Low dose (50mg/kg b.w.)	High dose (100mg/kg b.w.)
SOD (% inhibition /min/mg tissue)	3.8±0.2[@]	4.0±0.23	4.0±0.21
Catalase (µmole H_2O_2 liberate/ minute/ mg protein	7.7±0.14	7.5±0.17	7.2±0.12*
GST (µmoles of GSH / minute/ mg protein)	12.8±0.13	11.8±0.17*	11.8±0.36*
GPx (mM of GSH consumed/ mg tissue	13.2±0.2	12.4±0.15*	12.3±0.17*

Table 4.4. Induction of micronuclei in rat bone marrow due to subacute and subchronic exposure to thiacloprid

Group	% Micronuclei			PCE/NCE
	PCE	NCE	Total	
Subacute				
Control	1.7±0.04[@]	0.92±0.03	2.63±0.03	0.98±0.010
Low dose (50mg/kg b.w.)	2.4±0.06*	1.0±0.05	3.46±0.07*	0.96±0.012
High dose (100mg/kg b.w.)	2.5±0.07*	1.0±0.04	3.5±0.081*	0.94±0.016
Subchronic				
Control	1.8±0.037	0.84±0.02	2.67±0.02	1.00±0.012
Low dose (50mg/kg b.w.)	2.48±0.058*	0.98±0.02	3.44±0.060*	0.93±0.013*
High dose (100mg/kg b.w.)	2.44±0.060*	1.2±0.04*	3.50±0.084**	0.94±0.015*

[@]Data expressed as Mean±SEM; n=5 for each group; *p≤0.05 and **p≤0.01

Table 4.5. Effects of subacute and subchronic thiacloprid exposure on metaphase chromosomes of rat bone marrow cells

Aberration	Control	Low dose (50mg/kg b.w.)	High dose (100mg/kg b.w.)
Subacute			
Chromatid Aberration	1.33±0.004[@]	1.38±0.007	1.41±0.021**
Gap	0.81±0.005	0.85±0.013	0.86±0.12*
Break	0.52±0.007	0.53±0.015	0.56±0.012
Chromosome Aberration	0.23±0.01	0.28±0.012*	0.30±0.018*
Fragmentation	0.23±0.01	0.28±0.012*	0.29±0.016*
Translocation	00.00	00.00	0.007
Polyploidy	00.00	00.00	0.02
Centromeric association	00.00	00.00	0.02
Subchronic			
Chromatid Aberration	1.75 ±0.004[@]	1.95±0.036*	2.02±0.048**
Gap	0.85±0.012	0.95±0.028*	0.97±0.03*
Break	0.9±0.012	1.0± 0.025*	1.0± 0.028**
Chromosome Aberration	0.26±0.012	0.51±0.026 [a]	0.52±0.021 [a]
Fragmentation	0.24±0.012	0.31±0.015*	0.32±0.16*
Translocation	0.01±00.00	0.19±0.016[a]	0.2±0.15[a]
Polyploidy	00.00	0.09±0.003 [a]	0.1±0.003 [a]
Centromeric association	00.00	0.04±0.007**	0.05±0.01**

Table 4.6. Comet assay parameters of the experimental groups showing thiacloprid induced DNA damage in rat bone marrow

Group	Comet assay parameters				
	Tail length(μm)	Tail length distribution	Tail movement	Tail movement distribution	% Tail DNA
Subacute					
Control	18.0±1.9[@]	12.8 - 22.6[$]	21.8±2.3	15.6 – 29.6	51.3±1.6
Low dose (50mg/kg b.w.)	30.3±3.3	20.9 - 39.2	35.4±4.3*	22.6 – 46.7	60.5±3.3
High dose 100mg/kg b.w.)	35.6±4.6**	23.3 - 48.6	44.8±3.1**	35.6 – 52.7	69.3±4.2**
Subchronic					
Control	26.7±3.1	20.8 – 38.5	21.8±2.9	12.6 – 30.6	43.1±2.5
Low dose (50mg/kg b.w.)	44.4±3.0*	34.8 – 51.3	49.6±5.5**	36.5 – 65.3	63.1±3.2**
High dose 100mg/kg b.w.)	53.1±6.4**	37.6 – 69.4	54.6±6.5**	38.6 - 70.9	63.2±4.0**

[@]Data expressed as Mean±SEM; [$]Expressed in μm; n=5 for each group; *p≤0.05; **p≤0.01; [a] p≤0.001

Figure 4.1. Effect of thiacloprid exposure on MDA level

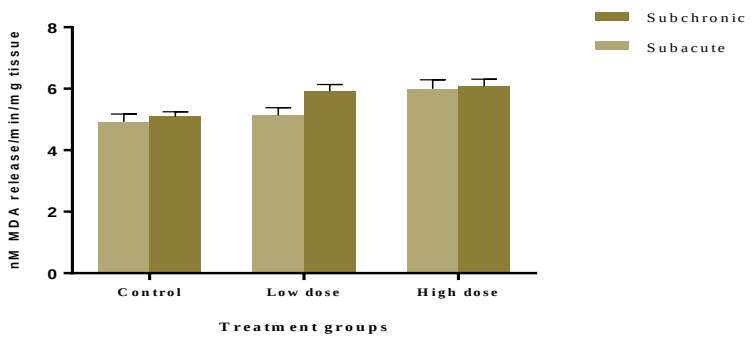


Figure 4.2. Effect of subacute thiacloprid exposure on ROS parameters

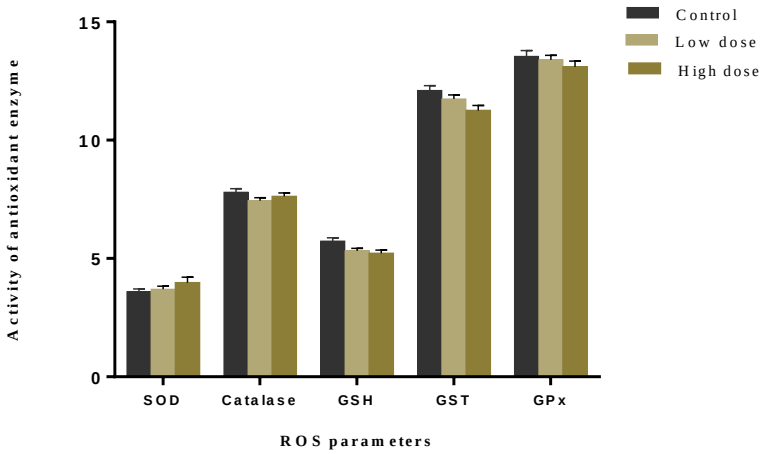


Figure 4.3. Effect of subchronic thiacloprid exposure on ROS parameters

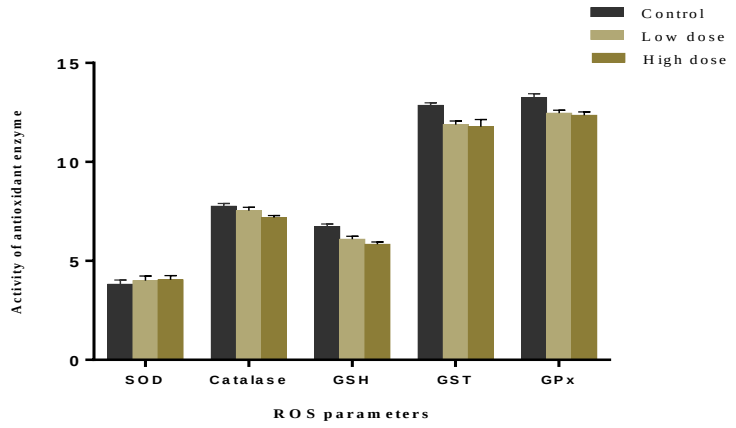


Figure 4.4. % Micronuclei in rat bone marrow cells after subacute and subchronic thiacloprid treatment

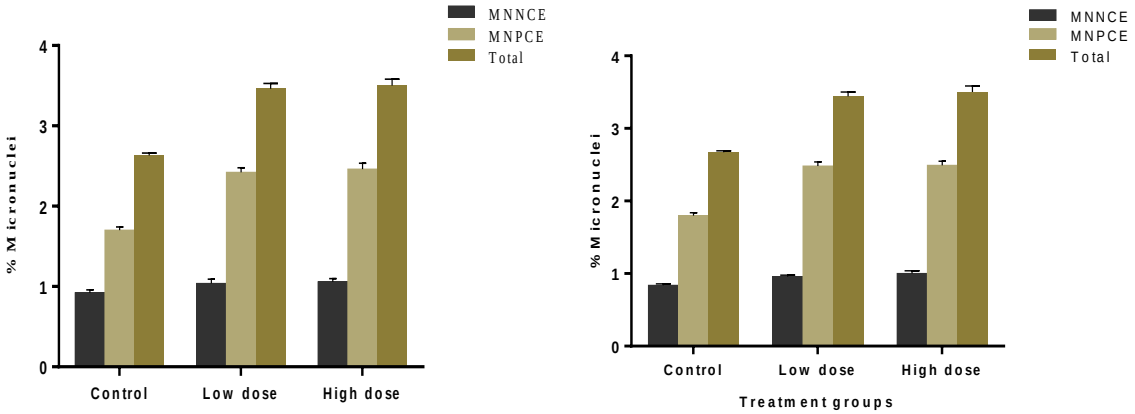


Figure 4.5. PCE/NCE ratio of the experimental groups

Figure 4.6a. Thiacloprid induced DNA damage after subacute exposure assessed by comet assay

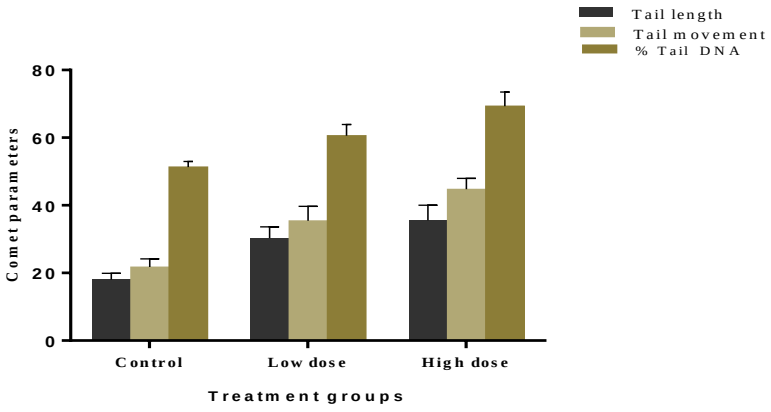


Figure 4.6b Thiacloprid induced DNA damage after subchronic exposure assessed by comet assay

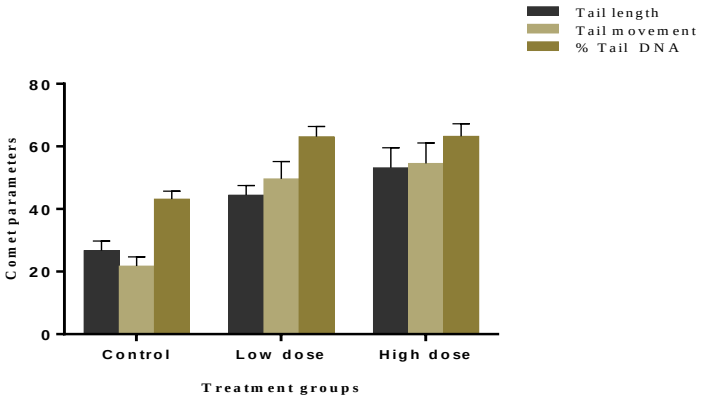


Figure 4.7a Thiacloprid induced chromosome aberration in rat bone marrow in subacute study

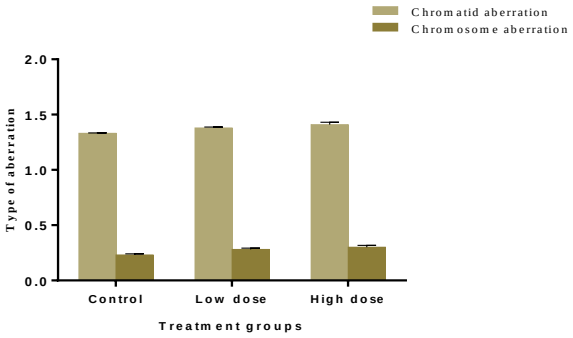


Figure 4.7b Thiacloprid induced chromosome aberration in rat bone marrow in subchronic study

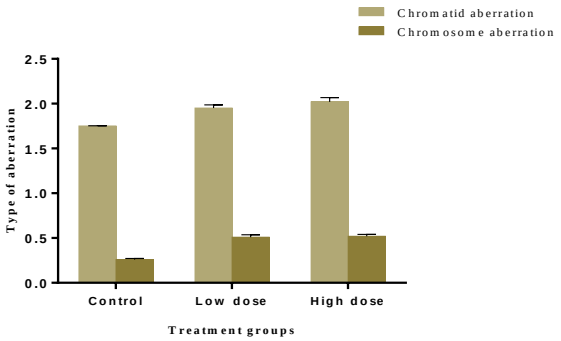
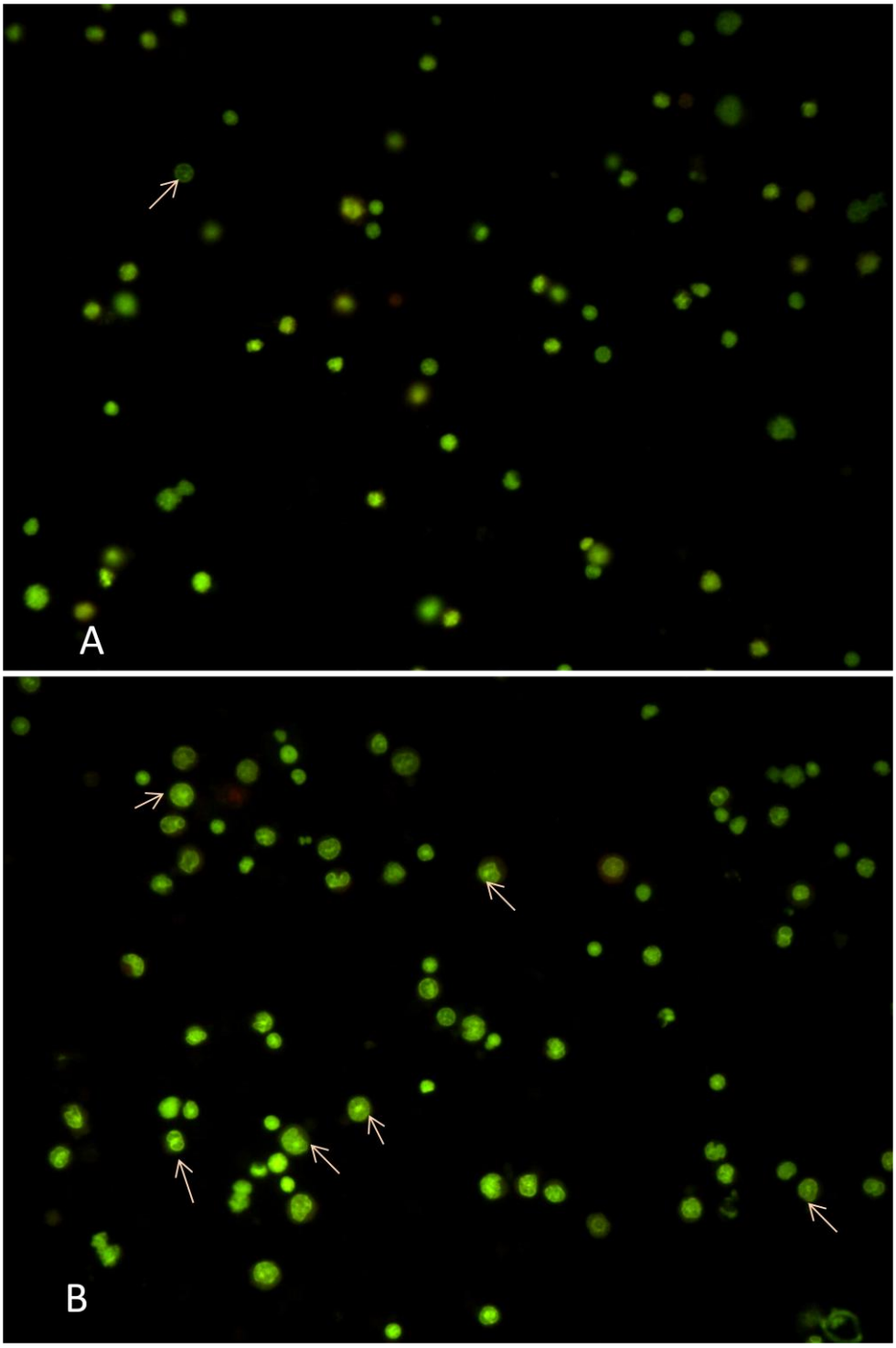
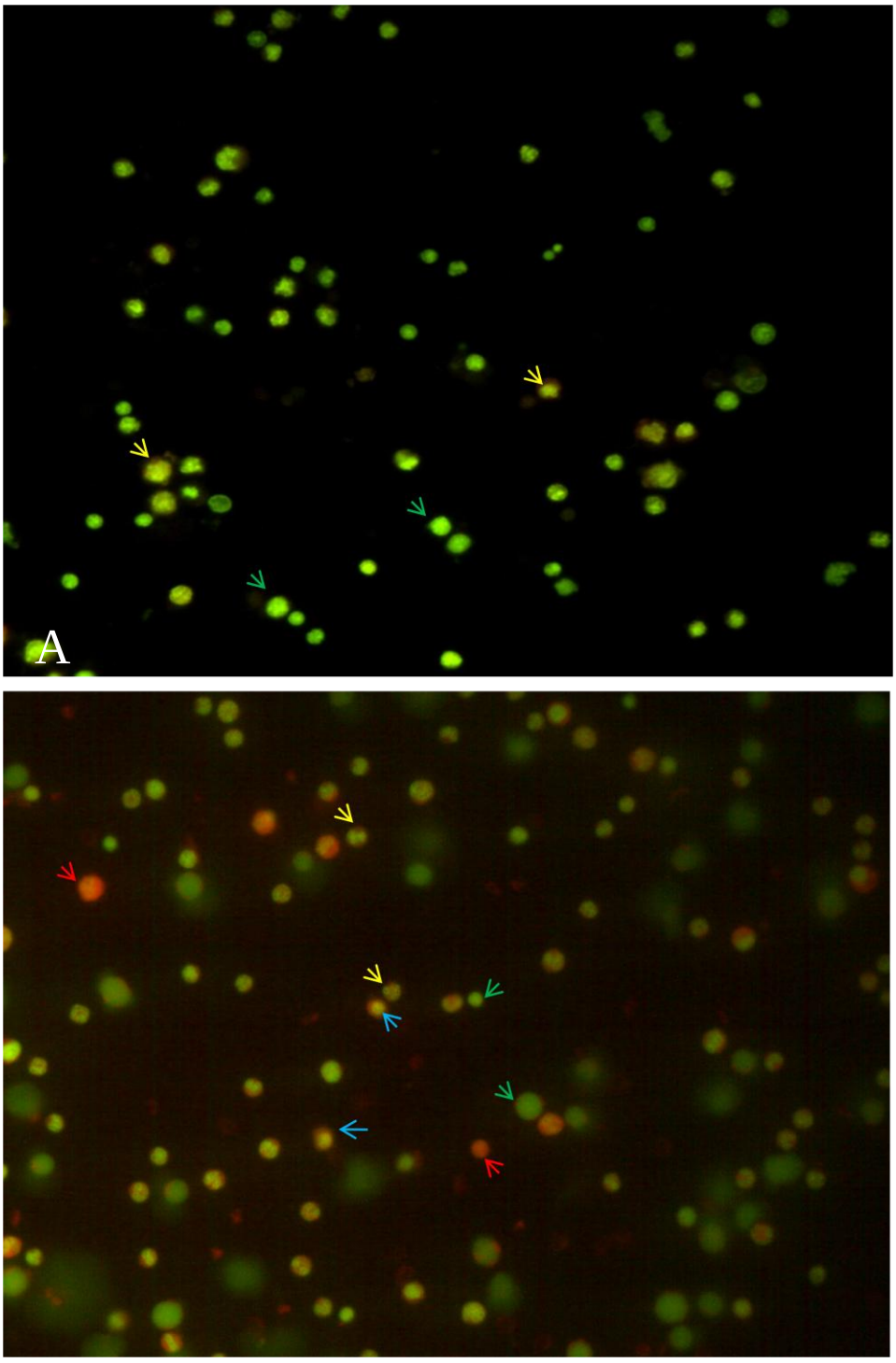


Figure 4.8 Fluorescence microscopy image of bone marrow cells of rat (Acridine orange staining, 20X).



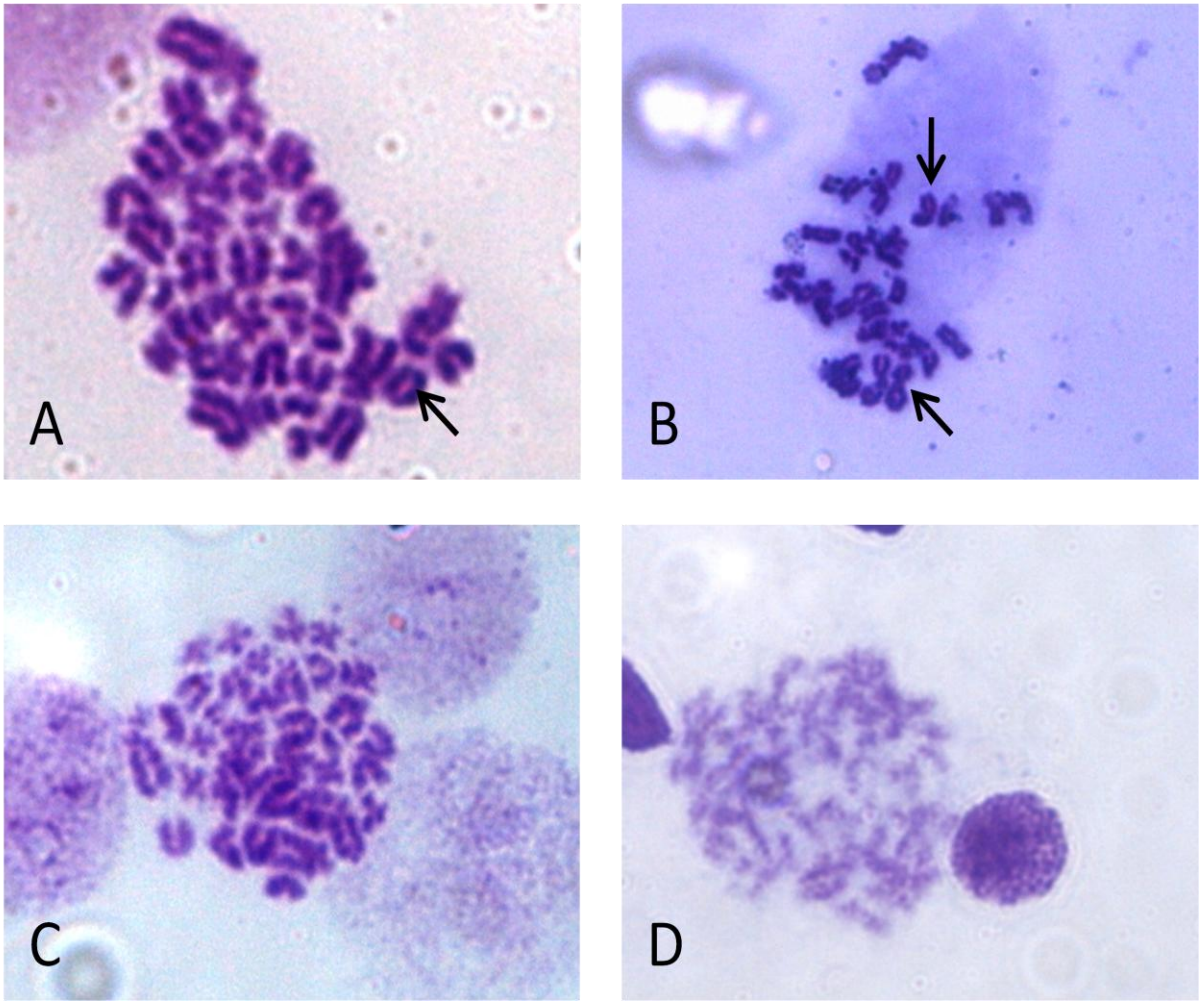
A). Bone marrow cells of control rat with one micronucleated cell. B). Bone marrow cells of thiacloprid treated many cell with micronucleus. Arrow indicates bone marrow cell micronucleus.

Figure 4.9 Fluorescence microscopy image of bone marrow cells of rat (Acridine orange and ethidium bromide staining, 20X).



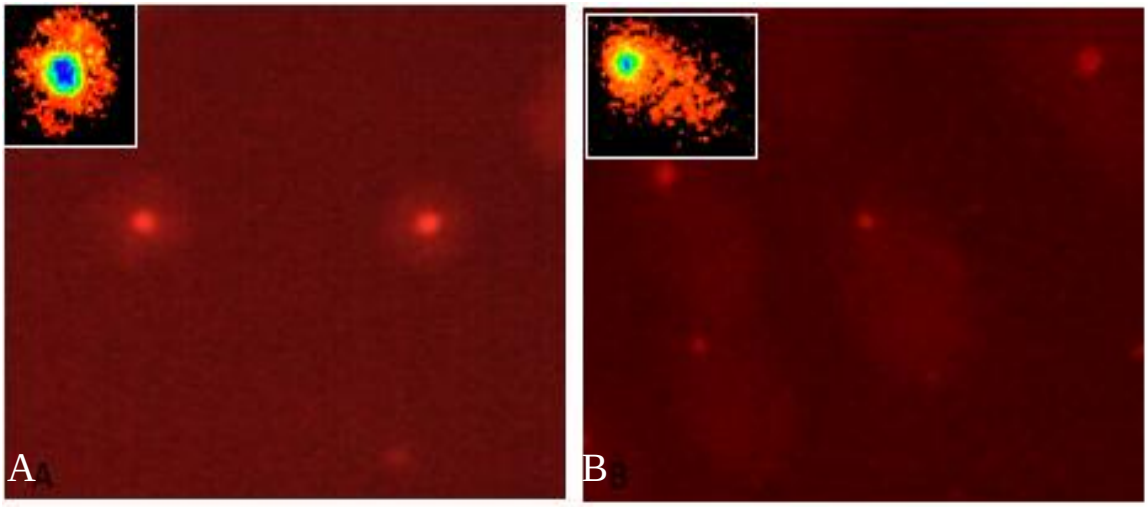
(A) Control bone marrow of rat with viable cell more in number (green arrow) and few apoptotic cell (yellow arrow). (B) Treated rat bone marrow with comparatively less viable cell (green arrow) and frequent apoptotic cell (yellow arrow early apoptotic cell and blue arrow late apoptotic cell). Necrotic cells were also marked (red arrow).

Figure 4.10 Chromosome aberration in thiacloprid induced rat bone marrow (Giemsa staining, 100X).



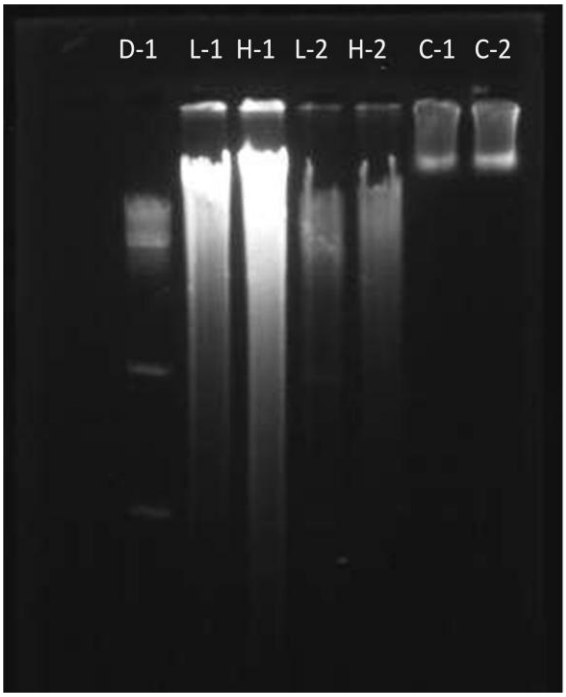
(A) Centromeric association (B) Dicentric association (C) Chromatid fragmentation fragments (D) Chromosome fragmentation.

Figure 4.11 Comet assay images showing comet tail length of rat bone marrow (20X).



(A) Control bone marrow comet tail length (B) Treated comet tail length of bone marrow cell. (CometScore analyzed photograph is on upright of both the figure).

Figure 4.12 DNA ladder assay for rat bone marrow cells.



1.4% agarose gel containing 10 μg of DNA/lane. DNA molecular weight marker (D-1), Subchronic low dose (L-1), subchronic high dose (H-1), subacute low dose (L-2), subacute high dose (H-2) and control.

GENERAL CONSIDERATIONS

The use of pesticides was first introduced in order to prevent, control and eliminate unwanted insects, pests and associated diseases. However, the increased use of these compounds has caused both, environmental and public health concerns (Bhanti *et al.,* 2007; Moore, *et al.,* 2010). Pesticides differ from other chemical substances because they are toxic chemicals deliberately spread into the environment with the aim of controlling undesired living species. Since their toxicity may not be completely specific for the target organisms, their use may pose a risk to human health. Pesticide poisoning remains a serious public health problem worldwide. More than 5 billion pounds of pesticides are used annually worldwide (EPA, 2004; Binukumar and Kiran, 2011).

Some of the most widely used pesticides are the organophosphates, which are found in many insecticides. Many are highly toxic and, due to their wide use, cause more poisonings and adverse effects on human health (Subash *et al.,* 2010; Mogda *et al.,* 2009). A search for highly insect-specific pesticides would prove to be more valuable and beneficial for human population. For this aim, Bayer AgroScience Ltd. came into the market with a new class of insecticides named neonicotinoids and registered with the U.S. EPA in 1982 Neonicotinoids bind to nAChRs of insects, mimicking nicotine, an alkaloid of tobacco plant. These compounds are classified by the EPA as both toxicity class II and class III agents and are labelled with the signal word "Warning" or "Caution" (Fishel, 2010).

Neonicotinoids, the newest major class of insecticides, have outstanding potency and systemic action for crop protection against piercing-sucking pests, and are highly effective for flea control on cats and dogs. They generally have low toxicity to mammals (acute and chronic), birds and fish (Tomizawa and Casida, 2005).

Thiacloprid, 3-(6-chloro-3-pyridylmethyl)-1,3-thiazolidin-2-ylidenecyanamide (IUPAC), is an insecticide of the neonicotinoid class registered with the U.S. EPA by Bayer AgroScience Ltd. Like the other neonicotinoids, thiacloprid shares structural similarity and a common mode of action with the tobacco toxin, nicotine (EPA, 2005). It displays selective toxicity for insects relative to mammals and displays a broad spectrum of useful properties. These properties include high insecticidal potency, control of insects resistant to the other major pesticides (e.g. organophosphates, carbamates and pyrethroids) and efficacy in soil application due to its

mobility from the roots to the upper parts of plants (Kagabu *et al*., 2002). Since being introduced in the insecticide market in 1985, the use of thiacloprid has increased each year. For the most part, neonicotinoid is replacing the acetylcholinesterase inhibitors, the organophosphorus compounds and methylcarbamates (EPA, 2002).

The toxicity of thiacloprid is based on the interference of neurotransmission in the nicotinic cholinergic nervous system. Thiacloprid binds to the nicotinic acetylcholine receptor (nAChR) at the neuronal and neuromuscular junctions in insects and vertebrates. The nAChR is an ion channel, of which the endogenous agonist is the excitatory neurotransmitter acetylcholine (ACh). The receptor normally exists in a closed state; however, upon ACh binding, the complex opens a pore and becomes permeable for cations. The channel openings occur in short bursts, which represent the lifetime of the receptor-ligand complex. ACh is then rapidly degraded by the enzyme acetylcholinesterse (AChE). In contrast, thiacloprid bound to the nAChR is inactivated very slowly. Prolonged activation of the nAChR by thiacloprid causes desensitization and blocking of the receptor and leads to paralysis and death (Matsuda *et al*., 2001; Matsuda *et al*., 2005).

Thiacloprid shows moderate acute toxicity after oral (LD50, 396–836 mg/kg bw) and inhalation (LC50, 1.223 to > 2.535 mg/L) exposure in rats, with females being more sensitive than males. Thiacloprid has low acute dermal toxicity (LD50 > 2000 mg/kg bw) in rats. Both the technical grade active ingredient thiacloprid and the end-use product of this Insecticide had health effects in animals when ingested and are considered to be potential skin sensitizers. Thiacloprid is not a skin irritant in rabbits, it was a slight eye irritant in rabbits, and it is not a skin sensitizer in guinea pigs also (EPA 2003, WHO 2009).

Health effects in animals given daily doses of thiacloprid over long periods of time included effects on the liver, thyroid gland, adrenal gland, testes and prostate gland. When thiacloprid was given to pregnant animals, effects on the developing foetus were observed at doses that were toxic to the mother, indicating that the foetus is not more sensitive to thiacloprid than the adult animal. Effects on reproduction were seen at doses that were highly toxic to adult animals. Thiacloprid is not genotoxic at acute exposure but does cause cancer in animals. Exposure over long periods of time causes DNA damage by forming DNA adduct molecules and leads to cancers of liver, thyroid gland, ovary and uterus in animals (PMRA 1995; PMRA 1996).

In animal studies, moderate to high doses have resulted in CNS stimulation, similar to nicotine, including tremors, impaired papillary function and hypothermia. There are few indications that thiacloprid causes damage to the nervous system of adult animals, but signs of structural changes in the brain are observed in developing animals exposed before and after birth (WHO 2009).

In the light of the above observations, it was thought pertinent to evaluate the potential toxic manifestations of thiacloprid using mammalian model. Alanto 240, 21.7%SC (Batch No. PGSC000002), a formulated product of thiacloprid manufactured by Bayer AgroScience Ltd. was selected and procured from the local markets of Vadodara. All experimental protocols were approved by IAEC (Institutional Animal Ethics Committee) in strict compliances with the guidelines of CPCSEA, India. Sprague Dawley (SD) male rats were procured from Sunpharma Advanced research Company Ltd. Akota, Baroda, which is a CPCSEA-approved animal breeder. Median lethal dose was calculated based on a pilot dose range study. For the present subacute and subchronic study doses of 1/15 and 1/30 of LD50 values were selected as per the widely accepted regulatory norms (OECD, 1998). Rats were randomized based on their body weight into three groups *viz.,* control group, low dose group (LTD) and high dose group (HTD). There were 5 animals in each group and the mean body weight of animals between the groups was by and large kept constant. Treated groups of animal were gavaged for 28 days for subacute and 90 days for subchronic evaluation of thiacloprid toxicity. All the experiments were conducted in strict adherence to the procedures of the Drugs and Cosmetics rules 1945, Appendix - III animal care standard.

On uptake by mammals, most neonicotinoids undergo metabolic alterations at multiple sites but liver is a major site for the metabolism of thiacloprid. Hence, the mechanistic study was designed and the hepatotoxicity by thiacloprid was assessed in SD rats (Chapter 1). Food consumption, body weight and behavioural changes were noted. Hepatotoxic potency of thiacloprid was estimated by liver marker enzymes and stress marker enzyme activity which was supplemented by histopahtological evaluation of liver tissue.

Food consumption and body weight of rats in treatment groups was slightly lower during the subacute exposure and significantly lower during subchronic exposure to thiacloprid. Observations made by Goyal (2010) during a toxicity study of thiacloprid in the digestive tract of birds opined that thiacloprid acts as an irritant to the intestinal membrane. He further suggested that this could reduce food consumption. Therefore, it is logical to presume that the

thiocloprid induced hampered mobility, general weakness and low food intake might have resulted in weight loss in experimental animals (Chapter 1). However, liver weight was found to be increased in the hepatotoxicity study of thiacloprid. Liver enlargement can occur as a result of changes in dietary composition or metabolic aberration. Liver enlargement without the accompanying histopathological change or functional impairment is often interpreted as being a physiological adaptation to enhanced workload or metabolic demand in body (Chopra and Griffin, 1985).

Serum AST and ALT are considered to be among the most sensitive markers employed in the diagnosis of hepatotoxicity. The currently observed heightened transaminase activity and decreased level of free radical scavengers were probably the consequences of thiacloprid-induced pathological changes in the liver and other visceral organs. Activities of serum enzymes like AST, ALT and ALP represent the functional status of liver (Mohany *et al*., 2011). High serum levels of AST and ALT are usually indicative of liver damage in animals (Durak *et al*., 1996) and humans (Ray and Drummond, 1991). Moreover, the increased transaminase activity could be a result of deranged carbohydrate metabolism an evident from increased LDH activity which is an index of anaerobic metabolism and a possible shift towards gluconeogenesis. It is well known that during gluconeogenesis liver transaminase activity steps up to convert amino acids like alanine to their respective keto acid for their ultimate conversion as glucose. Moreover, increased enzyme activity may possibly be based on mutation of genes for the synthesis of these enzymes as reported (Bolognesi and Morasso, 2000). Further, the increased LDH activity can also be used as an indicator of the potential of toxic agents to cause cellular damage (Bagchi *et al*., 1995).

Estimated value of protein was marginally higher for subacute study and significantly higher for subchronic study. Though, hike in serum protein level was reported in other studies involving xenobiotics, further careful experimentations at the transcriptional and/or translatory level needs to be conducted in order to conclusively comprehend such an observation. The Subacute exposure to thiacloprid did not cause any significant alteration in serum total bilirubin but the value was found decreased in case of subchronic exposure. Glucose levels were found decreased in case of both the repeated dose studies.

Activity of LPO in the terms of MDA level was found increased whereas, the activity of antioxidant enzyme and GSH level were found decreased. The decreased activities of SOD, GPx, Catalase and GSH together with increased LPO activity may have led to free radical

toxicity during subchronic exposure to thiacloprid. Thiacloprid toxicity may also induce histopathological alterations in liver. Similar decreased activity of antioxidant enzymes and increased activity of LPO was reported for imidacloprid in rats (Kapoor, 2010; Duzguner and Erdogan, 2012). Moreover, marked degeneration of hepatocytes in thiacloprid exposed rat liver tissue was observed and also changes such as vacuolation and focal necrosis of hepatocytes were seen. Similar observations reported by others while studying hepatotoxicity of similar class of pesticides give credence to the present findings (Goyal *et al*., 2010; Bhardwaj *et al*., 2010; Kammon *et al*., 2010; Toor *et al*., 2012).

However, it is well known that toxicity induced by different xenobiotics may disturb the endocrine function. In order to check whether repeated dose thiocloprid intoxication induces endocrine dysfunction, we evaluated the thiacloprid-induced toxicity on thyroid gland of rats (Chapter 2). To observe the thiocloprid induced thyrotoxicosis, we estimated TSH and thyroid hormone in the serum of treated and reference groups of SD rats. The study was also supplemented by serum lipid profile tests like cholesterol, HDL-C, LDL-C and triglyceride. A light-microscopy study was also undertaken and that revealed the histopathological changes in the thyroid of rats subjected to thiacloprid orally.

In the current study, thiacloprid exposure for 28 days caused marginal increase in serum TSH and also a decreased level of fT_3 hormone at the dose of 100mg/kg body weight. No such significant change was observed at the dose of 50mg/kg body weight. These results find support from similar findings reported by Sekeroglu (2012), who observed significantly increased TSH level and decreased (though statistically non-significant) levels of fT_3 and fT_4 at 112mg/kg body weight of thiacloprid given as a single dose. A plausible explanation for this adverse effect of thiacloprid on the thyroid gland is its metabolism to nitroguanidine, an inhibitor of nitric oxide synthesis. It is known that nitric oxide plays a vital role in the function of the thyroid gland and therefore, its inhibition can be a cause of the hypothyroidism that has been observed in our results.

Results of the current investigation also showed significantly increased serum cholesterol concentration after 28 and 90 days of thiacloprid treatment at the dose of 100mg/kg body weight and also increased serum triglyceride level (Chapter 2). Thyroid hormone plays an important role in the metabolism of lipids (Miyamoto *et al*., 1997). Hypothyroidism is usually associated with an increased serum concentration of total cholesterol and lipoproteins (Pucci *et*

al., 2000). It is known that overt hypothyroidism is associated with increased fasting plasma cholesterol and triglyceride levels (Tulloch, 1974).

Serum HDL-C and LDL-C were significantly increased after thiacloprid administration for 90 days and levels of both were also comparatively higher in animals dosed at 100mg/kg body weight for 28 days compared to control group. The results of the present study find support from the NRA (2001) thiacloprid public summary. Nikkila and Kekki (1972) observed a moderate increase of serum triglycerides in hypothyroid condition (in humans), associated with a decrease in efficiency of triglyceride removal from plasma, which was attributed to a low lipoprotein lipase (LPL) activity (Lithell *et al*., 1981; Staels *et al*., 1990).

Histological evaluation of the thyroid gland showed that rats given 3 months oral exposure to thiacloprid led to structural abnormalities in the thyroid gland (Chapter 2). This was indicated by reduction of connective tissue in interfollicular space as well as by completely absent or scanty colloids in follicles. The number of follicular epithelial cells was increased, layers of which were extremely flattened in some areas of the gland. Hyperplasia of parafollicular cells was also evident.

Further, since the test compound is toxic to the insect nervous system, it may have a potency to induce neurological alteration in mammals. Hence it was felt necessary to study the neurotoxicity in a mammalian model, which has been described in Chapter 3. Neuronal damage induced by the thiacloprid was assessed by estimating two esterases of significance acetylcholinesterase (AChE) and neuropathy target esterase (NTE). A behavioral examination was also carried out for rats treated with the pesticide in question. The functional neuronal abnormalities might induce or stems from structural anomalies therefore, light and transmission electron microscopic observations were also attempted.

The study indicates that activity of AChE was higher in blood and plasma in the thiacloprid treatment groups in the subacute study, whereas the same was observed to be low in the subchronic study as compared to control values. However, these differences were not statistically significant (Chapter 3). Brain AChE activity in animals treated at the dose of 100mg/kg body weight in subchronic study was found to be significantly decreased compared to control rats. A similar trend was observed for animals given a lower dose during the subchronic study and higher dose during the subacute study. Histochemical localization of this enzyme in rat brain too showed a similar trend. Bhardwaj *et al*. (2010) also reported significant

decreased activity of AChE in the brain and serum of female rats given 90 days repeated exposure to imidacloprid at a dose of 20mg/kg body weight.

Activity of NTE was observed to be decreased in the treatment group of rats in both 28 and 90 days studies. However, the decrease was significant only for animals of high dose treatment group in the subchronic study. Similar studies about thiacloprid or any other neonicotinoid pesticide have not been reported till date. Nevertheless, as explained elsewhere the above results might be due to the neuronal damage induced by thiacloprid.

The present study on rats exposed to thiacloprid for 90 days showed decreased neuromuscular coordination, which was reflected as increased number of falls from the rotating rod as compared to the control animals. It was found that thiacloprid treated rats could not keep balance on the rod at 25 rpm. This observation could be correlated to the development of ataxia in thiacloprid exposed rats in the subchronic study (Chapter 3). Significant sensorimotor impairments similar to the current results have been reported in female rats after exposure to a single dose of imidacloprid (Barnard *et al*., 1971; Abou-Donia *et al*., 2008). It is reported that such neurobehavioral deficits may reflect dysfunction at multiple anatomical areas in the central nervous system. Brain injury or damage may also be the cause of abnormal sensorimotor coordination (Hamm *et al*., 1994). Cresyl violet staining showed lesser uptake of stain by the cerebral region of thiocloprid intoxicated rat brain, indicating scanty distribution of Nissl bodies at that locus which reflects neuronal damage.

Vacuolated myelin degeneration of neurons was observed in TEM images. NTE inhibition is also related to localized accumulation of lysolecithin, a known demyelinating agent and receptor-mediated signal transducer (Quistad *et al*., 2003). In the present study, the myelination status of the nervous tissue was assessed in the experimental animals. As expected, the thickness of myelin was found less in the treated group of animals compared to control neurons. Researchers have suggested that myelin degradation is induced via Ca^{2+} influx into myelin and subsequent activation of cytosolic phospholypase A_2 and calain, which break down the myelin lipids and proteins and ultimately lead to excessive stimulation of Ca^{2+}-dependent degradative pathways (Fu *et al*., 2007; Trapp and Stys, 2009).

AChE inhibition and NTE inhibition due to thiacloprid exposure also induced histopathological changes in brain, as could be concluded from the histological observations made for rat brain. Neuronal degeneration and pyknosis of Purkinje cells with loss of granules in granular layer of hippocampus and thalamus region of brain were the several pathological

changes observed in the brain of treatment group of animals (Chapter 3). The histopathological change observed in the brain of treated rats was discussed and correlated with the published reports (Nagata *et al*., 1996; Bhardwaj *et al*., 2010).

Attempts were also made to gather information regarding thiocloprid induced developmental neurotoxicity. *In vitro* study gives idea about possible change in developmental anomalies *in vivo*. We checked the effect of thiacloprid on proliferative cells of IMR 32 which is a neuroblastoma cell line (Chapter 3). We observed elongation of neurite processes and the proliferation rate of IMR cells after thiacloprid exposure. The findings of study suggest that thiacloprid is associated with the occurrence of early and late apoptotic/necrotic processes in IMR 32 human neuroblastoma cells and support the contention that pesticide-induced neuronal cell death leading to neurodegenerative disease may, at least in part, be associated with early and late apoptosis of neurons.

There was limited information available in literature on thiacloprid-induced genotoxicity and therefore, there was a need to further explore the cytotoxicity and genotoxicity potentials of thiacloprid. We aimed to study the effect of thiacloprid as a genotoxicant (Chapter 4). ROS parameter, micronucleus assay, chromosomal aberration and DNA damage were assessed to observe thiacloprid-induced genotoxicity.

MDA level was found increased during the subacute toxicity study and significantly increased during the subchronic toxicity study. Lipid peroxidation results in altered membrane function and production of toxic and reactive aldehydes, mainly MDA, which is capable of interacting with proteins or DNA, thereby promoting mutagenesis (Cheeseman, 1993; Toyokuni, 1996). Along with an increase in lipid peroxidation, the activities of several antioxidant enzymes such as catalase, GST, GPx and SOD were observed to be significantly lower in the plasma of rats administered with thiacloprid in the current study. These findings were supported by studies by Lopez *et al*. (2007) and Duzguner and Erdogan (2012).

The percent frequency of occurrence of micronuclei in bone marrow cells was found to be increased significantly in case of treatment groups. Furthermore, increased chromosomal aberrations too were noted in the treated group of animals. DNA damage induced by thiacloprid was measured by single cell gel electrophoresis which was noted to be significantly higher in the animals of high dose group of thiacloprid of both the studies (Chapter 4). Elevated levels of ROS induce oxidative stress, which leads to oxidative DNA damage and micronucleus formation, a probable mechanism of genotoxicity (Ritesh *et al*., 2011).

Micronucleus frequency was also observed to be significantly higher in human peripheral lymphocytes exposed to thiacloprid (300µg/ml, 48 hrs treatment period) in an *in vitro* study done by Kocaman *et al*. (2012). The chromosomal type of aberrations could also arise due to misrepair of lesions in the G_0 stage of circulating lymphocytes as well as derived aberrations from precursor cells in bone marrow and thymus, as suggested by Carrano and Natarajan (1988).

Decreased ratio of PCE/NCE was observed during the genotoxicity evaluation of thiacloprid. An increased number of polychromatic erythrocytes with micronucleus were observed in thiacloprid exposed rats as compared to the control group of rats. It is considered that a decrease in the ratio of polychromatic erythrocytes (PCE) to normochromatic erythrocytes (NCE) (P/N) in the micronucleus test is an indicator of bone marrow toxicity induced by mutagens. Decreased P/N ratio is suggestive of the impairment of the erythropoietic system of bone marrow which resulted into more number of denucleated NCE in bone marrow instead of these entering into peripheral blood stream. The P/N ratio is also said to be an important parameter to monitor progression/regression of cancer that is capable of affecting erythropoiesis in bone marrow (Gerashchenko *et al*., 2012).

Further, bone marrow cells were stained with acridine orange and ethidium bromide, which gives a picture of cell death in bone marrow cells. Bone marrow cells form treated animals were found more in apoptotic cell death state. To support this finding, we also performed the DNA ladder assay in 1.4% agarose gels. This was reflected in the fragmented DNA, which is characteristic of apoptosis.

The results presented in this study warrant the necessity of a further, more detailed testing of the genotoxicity of this pesticide. We also recommend the need for a permanent biomonitoring of subjects occupationally exposed to various mixtures of pesticides, in order to detect early cytogenetic biomarkers of xenobitic-induced poisoning and to prevent further induction of DNA lesions, which could induce neoplastic growth of damaged somatic cells. The incidence of DNA damage at low concentrations of these neonicotinoid insecticides is of great regulatory significance since the risk involved gets augmented manifold due to reported high levels of application of this insecticide in agricultural fields (Calderon-Segura *et al.,* 2012) than the concentrations assayed in this study.

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