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-ylideneacyanamide (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 acetylcholinesterase (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 fT₃ 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 fT₃ and fT₄ 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
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 imidaclorpid 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 imidaclorpid (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 thiacloprid 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 phospholipase 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 thiacloprid 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 G₀ 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.