Chapter 2

REVIEW OF LITERATURE
2.1. Assessment of Thyroid Axis Disruption

There is substantial evidence that early-life exposure (gestational and lactational) to the pesticides of the group organophosphate (Slotkin et al., 2013; Haviland et al., 2010), organochlorine (Espinosa et al., 2010) and dithiocarbamate (Axelstad et al., 2011) impair thyroid axis of adult. Most of these studies focused on the effect of single pesticide at doses higher than the environmentally realistic exposure. Individual pesticide exposure at environmentally relevant level may not cause adverse effects, but simultaneous exposure to more than one pesticide during early life even at low doses could be hazardous for their cumulating impacts. There are few studies reporting impairment of thyroid hormone and/or histological lesions to the thyroid gland on mixture EDC exposure (Bowers et al., 2004; Flippin et al., 2010; Wade et al., 2002). Exposure to mixture of pesticides is an environmentally realistic situation and raises the concerns about their potential combined impact on human health. Hence, there is a compelling need to explore the consequences of exposures to mixture of endocrine disrupting pesticides at environmentally relevant doses. Mancozeb, a fungicide of the group dithiocarbamate is established as a thyroid disruptor (Axelstad et al., 2011; Kackar et al., 1997). Neonicotinoid insecticide IMI though designed to selectively target insect nicotinic acetylcholine receptors, its mammalian toxicity also has been reported (Tomizawa and Casida, 2000). Recently, reproductive endocrine toxicity of IMI in laboratory rodents was reported (Bal et al., 2012). Thyroid disrupting potential of this insecticide has not been studied beside a preliminary report of thyroid lesion in rodents on high dose exposure (Eiben and Kaliner, 1991). In vivo effect of thyroid axis disruption was evaluated by assay of circulating levels of thyroid hormones triiodothyronine (T₃), thyroxin (T₄) and pituitary hormone TSH. Effect on thyroid gland weight and histopathology were also studied to substantiate the in vivo study.


2.2. Assessment of Metabolic Dysfunction; Body Weight Impairment

The prevalence of obesity has risen dramatically all over the world not only in the adult population but also in children and adolescent groups. Many serious illnesses such as Type 2 diabetes, heart and respiratory diseases and reproductive problems which are more frequently reported in obese group at old age are now being noticed in overweight juveniles (Manikkam et al., 2013; Regnier and Sargis, 2013). Recent scientific research implies that increasing exposure to environmental substances having endocrine disruptive activities is the generative roots of obesity (Janesick and Blumberg, 2011; Legler et al., 2011). There is substantial evidence suggesting that disruption of hormonal and neurotransmitter systems by environmental endocrine disrupting chemicals (EDC) cause chronic imbalance in energy homeostasis that result in obesity (Cheung and Mao 2012; Newbold, 2010). There is increasing evidence that interference of developmental programming by EDC during critical fetal/neonatal period contribute greatly to the increase in obesity and other components of metabolic syndrome not only in adult but also in young and adolescent groups (Thayer et al., 2012; Valvi et al., 2012). Pesticides as a group because of their wide scale intentional use may contribute greatly to risk factors of obesity as many of them confirmed as EDC. In addition to disruption of endocrine axes, some active ingredients of pesticides/insecticides may also interfere with neurotransmitter (e.g., organophosphate, carbamate, neonicotinoids and/or ion channels (pyrethroids) which might affect glucose homeostasis at dose levels where they are effective as pesticides (Franklin and Wollheim, 2004; Satin and Kinard, 1998). However, much less research has been done on early life exposure effect of pesticides on body weight gain and other related metabolic dysregulation (Lassiter and Brimijoin, 2008;
Slotkin, 2010). Extensive research on a number of EDC such as diethylstilbisterol/DES (Newbold et al., 2010), poly chlorinated biphenyls/PCB (Givens et al., 2007), poly brominated diethyl ether/PBDE (Harley et al., 2011) and bisphenol A (vom Saal et al., 2011) has well supported the fact that in utero and early developmental exposure cause obesity/body weight gain later in life. In view of the fact that pesticides as environmental EDC remain relatively unexplored for their effect on obesity, the present study was undertaken for assessment of effect on body weight of mice offsprings who as neonates were lactationally exposed to low dose of dithiocarbamate mancozeb (MCZ), neonicotinoid imidaclorprid (IMI) and commixture.
2.3. Assessment of Adrenal Axis Disruption

Pesticide-induced impairment of adrenal function in adult rodents has been reported for organophosphate chlorpyrifos (Hashim et al., 2009; Rosol et al., 2001; Yano et al., 2000), organochlorine DDT (Jeong et al., 2006; Wilson et al., 2009) and dithicarbamates (Caroldi and De Paris, 1995). There is substantial evidence that early-life exposure (gestational and lactational) to the pesticides of the group organophosphate (Seidler et al., 2011), organochlorine (Jonsson et al., 1995) and dithiocarbamate (Maranghi et al., 2003) impair adrenal axis of adult. As early-life stages of development (prenatal and postnatal) are sensitive to any kind of stressors (Charmandari et al., 2005; Chrousos and Gold, 1992; Habib et al., 2000) activation of the adrenal axis is much sustained during critical windows of development. The adrenal gland is more susceptible to toxic impact of pesticides as compared to the other components of stress axis because of its highly vascularized nature and lipophilic property (Orjan and Lizette 2002; Philip and David 2003; Rosol et al., 2001). Studies on adrenal toxicity mostly focused on the effect of single pesticide at doses higher than the environmentally realistic exposure. A single pesticide at environmentally relevant dose level may not cause adrenal toxicity, but simultaneous exposure to low dose of more than one pesticide during early life may adversely affect stress axis as a result of their cumulating effect and/or synergistic/antagonistic effects. There are few studies reporting mixture EDC induced impairment of adrenal hormone and/or histological lesions to the adrenal gland (Wade et al., 2002). Two contemporary-use commercial pesticides, mancozeb (MCZ), and imidacloprid (IMI) were evaluated for their effects on the adrenal axis that were exposed during postnatal vulnerable phase of development. Mancozeb, a fungicide of the group dithiocarbamate is
established as a thyroid disruptor (Axelstad et al., 2011; Kackar et al., 1997) and
developmental (Ksheerasagar and Kaliwal 2010; Farag et al., 2007) and reproductive
(Farag et al. 2007; Sayim 2007) toxicity of mancozeb has been documented in rodents.
The widely used neonicotinoid insecticide IMI though designed to selectively target
insect nicotinic acetylcholine receptors, its mammalian toxicity also has been reported
(Tomizawa and Casida, 2000). Recently, IMI was reported to cause reproductive
endocrine toxicity in laboratory rodents (Bal et al., 2012). Adrenal disrupting potential of
this insecticide on low dose exposure has not been studied in both pesticides. Adrenotoxicity were not evaluated of exposure of this individual as well as mixture
pesticides at early life. The adrenotoxic effects were investigated in neonates (PND 29) of
both the sexes who were lactationally exposed to pesticides. The persistence of the
adverse effects was further checked in young adult mice (PND 63).
2.4. Assessment of Hippocampal Toxicity and Behavioral Abnormalities

The hippocampus belongs to the limbic system which is located in medial temporal lobe of brain. It plays important role in forming new memories, emotional response, senses navigation, spatial orientation and modulation of short-term memory to long-term memory (Kesner and Gilbert, 2004; O’Keefe, 1999; Rusakov et al., 1997). It shows an impressive capacity for structural reorganization undergoing modifications in neural connections, synapse number and dendritic complexity. It has four anatomical subdivisions, CA1, CA2, CA3 and CA4 (CA: cornu ammonis); CA1 and CA3 have larger regions and more neural connections than CA2 and CA4 (Amaral et al., 2006). The major pathways of neuronal signal flow from CA1 and CA3 region to other parts of brain which are densely filled with pyramidal neurons. The dendrites of pyramidal cells have larger diameter than other neuronal types with high number of spine density, and synaptic inputs (Gulyas et al., 1999; Megias et al., 2001). Pyramidal neurons in CA1 and CA3 areas undergo dynamic modifications in the form of dendritic extension and retraction, as well as in synapse formation and elimination which occur till adolescence (Casey et al., 2008; Bremner and Vermetten, 2002). Hippocampus is a very sensitive part of brain, structural organization and functionality of which gets affected by environmental pollutants, such as pesticides, metals and plasticizers (Modgil et al., 2014; Cui et al., 2006; Douglas et al., 2007). Pesticides, the wide range of chemicals in applications in croplands and household to control weeds, insects and fungi, are reported to alter cytoarchitecture of pyramidal neurons of CA1 and CA3 regions and impair physiological functions of hippocampus (López-Granero et al., 2013; Ahmed et al., 2013; Astiz et al., 2013; Liu et al., 2013). Pesticides are also reported to interfere with proliferation,
differentiation, synaptogenesis and myelination of neurons when exposure was done during the critical phases of brain development (McEwen and Magarinos, 2001; Jiang et al., 2014; Guilarte et al., 2012). Neonatal exposure (postnatal day/PND 6 to 21) to permethrin (pyrethroids pesticide) causes alteration of synapse morphology in hippocampus in rat (Nasuti et al., 2014). Maternal dietary exposure (gestation day/GD 10 to PND 21) to chlorpyrifos (organophosphates) impaired hippocampal neurogenesis in male mice (Wang et al., 2013). Dendritic branching pattern of CA1 pyramidal neurons and spine density were affected in neonatal rats of PND 21 and PND 28 when exposed subcutaneously to organophosphate paraoxon from PND 8 to PND 20 (Santos et al., 2004). Besides these limited studies on structural plasticity of hippocampus, early-life exposure effect of pesticides on neurotoxicity, behavioral alteration including impairment of learning-memory has also been reported. Early life exposure to dithiocarbamate mancozeb (MCZ) induced neurotoxicity at and above 150 mg/kg bw/day and its metabolites propyl thiouracil impaired learning and memory in rodents (Axelstad et al., 2011; Axelstad et al., 2008). Neonicotinoid insecticide imidacloprid (IMI) also cause developmental neurotoxicity and behavioral alteration including impairment of learning-memory in prenatally exposed offsprings (Abou-Donia et al., 2011). Early life exposure of MCZ along with mixture of other pesticides (epoxiconazole, prochloraz, tebuconazole and procymidone) induced impairment of learning and memory in rat (Jacobsen et al., 2012). Individual exposure of many of these pesticides including MCZ, however could not affect the learning-memory ability in offspring when exposed at dose-level higher than in mixture (Vinggaard et al., 2005; Axelstad et al., 2011; Moser et al., 2001; Christiansen, 2009). Inspite of the fact that in the environmentally realistic situation every
organism is susceptible to mixture of pesticides rather than individual ones, studies on the
effect of combinatorial exposure of pesticides on plasticity of hippocampus have not been
explored. The present investigation was carried out to elucidate the hippocampal
plasticity of mice lactationally exposed (PND 1–PND 28) to MCZ and IMI as individual
as well as combinatorial exposures. These two pesticides are currently in use as a
fungicide and insecticide respectively for the protection of crop thereby making the
human beings/wild animals susceptible to their exposures. The impacts of exposures were
assessed at two points. Effect of immediate exposure was examined in the neonates of
PND 29 and effect was further analyzed at sexual maturity age of PND63 to address
whether the adverse effects observed, if any, persistent to adulthood or not. MCZ and IMI
induced effect on structural alteration of hippocampus was evaluated through study of
modifications in pyramidal neurons of CA1 and CA3 regions. An attempt also has been
made to correlate the neuronal modification to pesticides induced alteration of plasma
level of thyroid (T3, T4 and TSH), adrenal (corticosterone) and gondal (testosterone)
hormones. Recently we reported that combinatorial exposure to MCZ and IMI during
early life induced hypothyroidism in adult mice (Bhaskar and Mohanty 2014). Thyroid
hormones play key roles in the development of the brain, particularly shape and size of
neuronal cells, neuronal migration and dendritic arborization of pyramidal neuron of the
hippocampus as well as behavior (Brito and Moura 1997; Higueret and Enderlin 2008;
Gerges and Alkadhi 2004). Besides thyroid hormone, hippocampal development and
function are also exquisitely sensitive to the glucocorticoids and gondal hormone.
Glucocorticoid hormone has important role in structural plasticity of CA1 and CA3
regions and learning-memory as there is abundance of glucocorticoid receptors in CA1
and CA3 regions of hippocampus (Morimoto et al., 1996; McEwen and Lasley 2002; Kim and Diamond, 2002). Gonadal hormones plays crucial role in brain maturation and behavior during the adolescence period (Sisk and Zehr 2005). It has role in neuronal modification in the CA1 and CA3 field of hippocampus which significantly contributes to learning and memory ability in adolescence animals (Leranth et al., 2003; Isgor and Sengelaub, 1998). The implication of hippocampal alteration on learning-memory deficits in mice was elucidated through behavioral analysis.
2.5. *In Silico* Assessment of Pesticides by Molecular Docking and QSAR Methods

Over the past decades, growing body of evidences suggest that numerous xenobiotics may interfere with the endocrine system and produce adverse effects in humans as well as wildlife. These xenobiotics are known as Endocrine Disrupting Chemicals (EDCs). Many of these substances have been linked with developmental, reproductive, neural, immune, and increased incidences or progression of some diseases, including obesity, diabetes, endometriosis, and some cancers in animals. EDCs have various heterogeneous compound such as polychlorinated biphenyls (PCBs), polybrominated biphenyls (PBBs), bisphenol A (BPA), plasticizers (phthalates), pesticides (methoxychlor, chlorpyrifos), and pharmaceutical agents diethylstilbestrol (DES). EDCs have identified nature of estrogen, androgen, anti-androgen, progesterone, or thyroid-like activity. There are several possible modes of endocrine disruptive actions. The important mode of EDCs action can affect possible cellular hormonal pathway; most information is available about interference of EDCs with the endocrine hormone receptors. The hormone receptors of this family are the gonad hormone receptors, androgen receptors (ARs), progesterone receptors (PRs), glucocorticoid receptors (GRs), and mineralocorticoid receptor (MRs) as well as the thyroid hormone receptors (TRs). Some EDCs can bind directly to these receptors either as agonists or antagonists, thus enhancing or inhibiting the effect of a hormone. Direct interactions with estrogen receptors are well reported for the phthalates, alkylphenols, bisphenol A, chlorinated pesticides such as DDT and methoxychlor (Nicolopoulou-Stamati and Pitsos, 2001; Wolf et al., 2004; Cooper and Kavlock, 1997). PBDE, PCBs and bisphenol A are shown binding nature with thyroid receptor (Gentilcore et al., 2012;
Moriyama et al., 2002). Polyhalogenated bisphenols, including the Tributyltin (TBT), are disrupters of PPAR and RXR receptors signaling (Riuet et al., 2011; Maire et al., 2009). Other important mechanisms are EDCs mediated interaction with hormone metabolism. EDCs have been described to interfere with all of these processes (Baker et al., 1998; You et al., 2001, Boas et al., 2006). Steroid/thyroid hormone metabolism is particularly affected by EDCs. Cytochrome P450 (CYP) enzymes have a key function in the synthesis and degradation of steroid hormones, and their production or activity can be influenced by EDCs. It is possible for certain EDCs to cause or contribute to hormonal disruption and subsequent reproductive and developmental toxicities by interfering with the function of key enzymes involved in steroid synthesis and breakdown (Miller, 1988).

For example, Persistent organochlorines, azole as well as organophosphates are known to alter cytochrome P450 (CYP) enzyme activity (Walsh et al., 2000; Walsh and Stocco 2000; Sharara et al., 1998). Pesticides such as TBT, DDE impairment of Cytchrome P450 functioning were also reported (Fernandes et al., 2007). The CYP450 (CYP1A2, CYP3A4, CYP1A1, CYP11B1 and CYP11B2) metabolism could play a significant role to evaluate endocrine toxicity.

The toxicological evaluation of chemicals/pescidides using in silico techniques, such as molecular docking and QSAR (Quantitative Structure Activity Relationships), aims to reduce the amount and time for toxicological evaluations and could be applied to the prediction of endocrine disruption, ecological toxicity as well as bioaccumulation toxicity. QSAR tools predict the ecological toxic effect (Fathead minnow LC<sub>50</sub>, Daphnia magna LC<sub>50</sub> and Tetrahymena pyriformis IGC<sub>50</sub>), Bioaccumulation toxicity (ratio of the chemical concentration in fish as a result of absorption via the respiratory surface to that
in water at steady state), human developmental toxicity (a chemical causes developmental toxicity effects to humans or animals) and Ames mutagenicity.

Keeping in view the importance of the study aspects mentioned above, the present investigation was carried to explore toxicity and to predict endocrine disrupting potential with the aim of comparatively evaluating to mancozeb and imidacloprid.