CHAPTER 1
INTRODUCTION

Organophosphate pesticides (OPPs) are globally used in modern agriculture, industries and household for pest-control purposes, public health and for prevention of infectious diseases. They are extensively used in agriculture for pest-control purposes to increase the productivity of crops to meet the demand of escalating population (Jaga et al., 2003). However, their persistent exposure poses threat to environment as well as human health. OPPs poisoning is a leading cause of morbidity in developing countries (Buckley et al., 2004). OPPs poisonings is more prevalent among the agricultural workers, manufacturing workers, and small children (O’malley, 1997). Substantial exposure of OPPs has been speculated to be associated with various human pathologies including cancer (Alavanja et al., 2012; Chen, 2012). Apart from their neurotoxic nature as acetylcholinesterase (AChE) inhibitors (Kwong 2002), OPPs have been also reported to induce oxidative stress and cause oxidative damage to the cells. Studies have indicated that OPPs stimulate oxidative stress (Abdollahi et al., 2004; Shadnia et al., 2005); that may contribute to the chronic inflammation and carcinogenesis (Federico et al., 2007). Mostly used OPPs are chlorpyrifos (CP) and monocrotophos (MCP). Genotoxic potential of CP and MCP in human health has been well explained. Various studies using in vitro and in vivo models have shown that CP and MCP induce generation of ROS, apoptosis, oxidative DNA damage, chromosome aberrations and alterations in cell-cycle (Bebe et al., 2003; Chauhan et al., 2016; Kashyap et al., 2011; Kashyap et al., 2010; Salazar-Arredondo et al., 2008). MCP has been shown to induce MCF7 breast cancer cell proliferation (Isoda et al., 2004).

Oxidative stress takes place when the ROS generation exceeds the antioxidant system of the cell. ROS are free radicals which are highly reactive atoms or molecules containing one or more unpaired electrons in their outer shell. ROS are constantly formed during normal cellular processes, such as during energy production, metabolic processes or by auto-oxidation of molecules (Bayr,
Abnormally high ROS levels can cause oxidative damage to cell macromolecules viz. lipids, proteins, and nucleic acids, as well as causes the dysregulation of oncogenes and tumor suppressor genes. Considering that the toxicity of OPPs has been associated with the production of ROS which is implicated in the process of carcinogenesis; our study intends to determine the cellular signalling associated with the cell survival against genotoxic effect of OPPs CP and MCP in A549, NCI-H23 and NCI-H1299 non-small cell lung carcinoma cell lines as a model study.

Lung cancer cause was chosen for the present study because it displays the highest incidence as well as mortality rates worldwide (Torre et al., 2016). According to American cancer society, 1 out of 4 cancer deaths are due to lung cancer which is more than any other cancers such as breast, colon, prostate etc. (www.cancer.org.). The risk of development and progression of lung cancer is more in the presence of environmental factors OPPs. Among the routes of pesticide absorption, the onset of symptoms and damaging effect is quickest when followed respiratory route via inhalation. Dermal absorption is relatively slow and oral ingestion is either accidental or suicidal (Kwong, 2002; O'malley, 1997). Therefore, lung cells are most vulnerable to the environmental factors OPPs-induced carcinogenesis process. Lung cancer is classified into two main types, non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). NSCLC is more common and the only lung cancer associated with non-smokers. For NSCLC, surgery is the prime therapy of choice in stage I–III tumors. However, in advanced stage IV tumors, only palliative care remains the option. In fact, most of the lung cancer cases are inoperable with poor survival rate (Ettinger et al., 2012). Late advanced stage diagnosis and limited options of therapeutic interventions advocate for a much-focused research for lung cancer, especially, when unavoidable environmental conditions pose as one of the major risk factors. For prevention and cure, we need complete understanding of molecular mechanisms underlying OPPs genotoxic potential.

OPPs potentially induce ROS, ROS-mediated cell signalling pathways and DNA damage response pathways are the prime choice of the investigation. Against oxidative DNA damage, base excision repair (BER) pathway is the
predominant DNA repair pathway in mammalian cells. DNA BER-pathway involves five to six major enzymes that act in a coordinated fashion to complete the repair process. BER-pathway is of two types, single nucleotide (SN) or short-patch (SP)-BER and long-patch (LP)-BER, depending upon the length of the repair patch. Both BER sub-pathways use different set of enzymes. SN-BER starts by the first enzyme monofunctional DNA glycosylase (DG) such as Thymine DNA glycosylase (TDG) that recognizes and removes the damaged base generating an AP site. The key regulator enzyme AP endonuclease 1 (APE1) then incises the phosphodiester backbone 5’ to the AP site generating 3’-OH and 5’-deoxyribosephosphate (dRP) termini. DNA polymerase beta (pol β) first catalyzes the excision of the 5’-dRP group via its dRP lyase activity and then fills the single nucleotide gap using its DNA polymerase activity. The downstream BER proteins such as X-ray repair cross complementing group 1 (XRCC1) acts as a scaffold, XRCC1-DNA ligase IIIα complex then seals the nick and completes the repair process as extensively reviewed (Hegde et al., 2008; Thakur et al., 2014).

In LP-BER sub-pathway, bifunctional DGs viz. 8-Oxoguanine DNA glycosylase (OGG1), Nei endonuclease VIII-like 1 (NEIL1) removes the damaged base as well as incises the AP site due to its additional AP lyase activity, which generates 3’-4-hydroxypentanal phosphate terminus. APE1 then removes this 3’-4-hydroxypentanal phosphate terminus to generate a 3’-OH group. DNA pol β fills in the gap and DNA Ligase IIIα seals the nick. LP-BER sub-pathway results in a repair patch that is 2–12 nucleotides long. LP-BER sub-pathway involves other additional proteins such as proliferating cell nuclear antigen (PCNA), replication factor C (RFC), flap endonuclease 1 (FEN1), DNA pol delta (δ) and epsilon (ε), and DNA ligase I to complete the repair process (Sweasy et al., 2006).

Dysregulation in BER-pathway has been associated with cancer (Maynard et al., 2009; Tudek 2007). The reduced DG OGG1 activity has been linked with lung cancer risk (Paz-Elizur et al., 2003). Altered APE1 expression has been linked with prostate and lung cancer (Kelley et al., 2001; Yoo et al., 2008). DNA pol β has been reported to be over-expressed in various cancers including lung cancer (Albertella et al., 2005). APE1 is a master regulator enzyme of BER-pathway with pleotropic role in cellular response to oxidative stress. Its
endonuclease activity in DNA repair function takes place via C-terminal domain; whereas, the N-terminus includes the nuclear localization signal (NLS) region which, through Cys65, mediates the regulatory function of APE1 as reductive-activator and redox-co-activator of various pro-survival transcription factors (TFs) such as activator protein 1 (AP-1), nuclear factor κ-B (NF-κB) and nuclear factor, erythroid-derived 2, like 2 (Nrf2) involved in cell proliferation, differentiation, angiogenesis, and anti-oxidant system (Bhakat et al., 2009; Fishel et al., 2015; Tell et al., 2009). Owing to multifunctional nature of APE1 in various cellular biological functions, it may not be confounding that APE1’s altered expression levels and functions have been linked with various cancers (Kelley et al., 2001; Woo et al., 2014), including lung cancer (Sevilya et al., 2015; Yoo et al., 2008).

One of the important factors inducible by ROS and redox-regulated by APE1 is c-jun, which is a component of TF AP-1. Study has shown that APE1 reduces the Cys272 residue in the c-jun DNA-binding domain and stimulates the DNA-binding activity of AP-1 (Xanthoudakis & Curran, 1992; Xanthoudakis et al., 1992). AP-1 is involved in cell proliferation, cell growth, apoptosis and transformation (Shaulian et al., 2002). ROS-induced JUN kinase (JNK) phosphorylates Ser63/73 at the N-terminal transactivation domain to activate c-jun; phospho-c-jun (p-c-jun) activates the c-jun transcription. Another potential target of oxidative stimuli is NF-κB, whose DNA binding activity is also stimulated through reductive activation by APE1. NF-κB is employed in regulation of genes involved in inflammation, cell proliferation, metastasis and apoptosis (Dolcet et al., 2005). Hence, the functional consequences of TF AP-1 and NF-κB activation as well as their downstream signalling mechanisms lead to carcinogenesis (Dolcet et al., 2005). Another TF which is known to be regulated by APE1 is Nrf2. Nrf2 binds to anti-oxidant response element (ARE) in promoter region of anti-oxidant and cytoprotective enzymes at the gene level and plays a role in carcinogenesis (Homma et al., 2009; Sporn et al., 2012; Tian et al., 2016). APE1 stimulates the DNA binding activity of these three TFs c-jun, NF-κB and Nrf2 in a redox-dependent manner. Alongwith ROS, reactive nitrogen species (RNS) is also involved in the process of carcinogenesis. RNS from nitric oxide (NO) can modify proteins and their functions. Inducible nitric oxide synthase (iNOS/NOS2)
generates large amount of NO and iNOS/NOS2 is induced by induced by cytokines viz. IL-1, TNF-α, and lipopolysaccharide [LPS] (Reuter et al., 2010).

After APE1’s altered expression, DNA repair i.e. endonuclease and redox-regulatory functions in response to oxidative stress, changes in APE1’s subcellular localization is another important aspect because subcellular localization defines the function. The primary localization of APE1 is nuclear, which controls cell proliferation rate (He et al., 2003). However, cytoplasmic localization has been also observed in some cell types. Increased cytoplasmic expression has been associated with various tumorigenic processes (Tell et al., 2005), particularly lung cancer (Puglisi et al., 2001).

Oxidative stress has been also documented to modulate the process of apoptosis. Any defect in apoptosis can modulate the cancer risk (Mashima et al., 2005). The key effectors of apoptosis are caspases that can be activated by intrinsic and extrinsic stimulus. ROS initiates the intrinsic pathway and results in the mitochondrial release of pro-apoptotic factors such as cytochrome c, apoptosis-inducing factor (AIF), and others. Released cytochrome c combines with the apoptotic activator factor-1 (Apaf-1) and interacts with initiator caspase-9 to form apoptosome, and finally triggers the activation of effector caspases-3, caspase-6, and caspase-7, leading to apoptosis (Kroemer et al., 2007). Released AIF when translocate to the nucleus, it causes condensation of chromatin in the nuclei and DNA fragmentation. However, mitochondrial AIF has been reported to have a pro-survival role (Daugas et al., 2000). Other factors related to apoptosis are Bax and Bcl-2. Bcl-2 is anti-apoptotic and its over-expression has been reported in cancer (Amundson et al., 2000); whereas, Bax favors apoptosis.

The knowledge gaps are:

- There is an inadequate evidence for the genotoxicity of CP and MCP in humans. The genotoxic effects of MCP and CP in association with lung cancer risk need to be evaluated at the molecular level.
- Cellular responses to oxidative stress and DNA damage after exposure to CP and MCP.
Functional characterization of APE1, at the biochemical and cellular level has not been performed so far in lung cancer cell lines viz. A549, NCI-H23 and NCI-H1299. Role of APE1 in regulation of cellular response to oxidative stress and DNA damage after exposure to CP and MCP need to be determined.

**Objectives of the study:**

1. To evaluate the extent of oxidative damage by pesticides (CP and MCP) on lung cancer A549, NCI-H23 and NCI-H1299 cells.
   1.1. OPPs-induced oxidative stress and subcellular localization of APE1.
   1.2. OPPs-induced changes in DNA content.

2. To study the pesticide-modulated altered cellular functions of APE1.
   2.1. Estimation of cellular expression levels of APE1 after exposure to MCP and CP.
   2.2. DNA repair pathway analysis.
   2.3. To elucidate the redox role of APE1 in regulation of transcription factors.
   2.4. APE1’s protein-protein interactome analysis.

In the present study, it is hypothesized that oxidative stress generated by OPPs CP and MCP results in oxidative DNA damage to which the cellular responses involve changes in DNA BER-pathway and cell survival signalling pathways; also, modulates BER-pathway’s key enzyme APE1’s other functions, i.e. redox-regulation of cell survival TFs, that may ultimately lead to increased risk for lung cancer progression.