Review of Literature
2.1. Carcinogenesis

Carcinogenesis or oncogenesis is literally the creation of cancer. It is a process by which normal cells are transformed into cancer cells. It is characterized by a progression of changes on cellular and genetic level that ultimately reprogram a cell to undergo uncontrolled cell division, thus forming a malignant mass. Cancer is a popular generic term for malignant neoplasms, occurring in all human and animal populations and arising in all tissues composed of potentially dividing cells. The basic characteristic of cancer is the transmissible abnormality of cells that is manifested by reduced control over growth and function leading to serious adverse effects on the host through invasive growth and metastases. Although progress has been made in reducing incidence and mortality rates and improving survival, cancer still accounts for more deaths than heart disease in persons younger than 85 years (Jemal et al., 2010). Studies conducted using animal models, “in vitro” studies and epidemiologic assays enabled investigators to conclude that neoplastic pathogenesis is a complex process which can be divided into three distinct stages, from an operational point of view. These are: initiation, promotion and progression (Gutierrez and Salsamendi, 2001; Trosko, 2001). Changes in the genome’s structure occur across the three stages of neoplastic development (Pitot, 2001; Luch, 2005). Changes in gene expression also take place during the promotion stage, with selective proliferation of initiated cells and the development of pre-neoplastic cells (Grisham et al., 1984; Gutierrez and Salsamendi, 2001). During initiation and promotion, apoptosis and cell proliferation can occur at different rates, while remaining balanced. During progression, this balance is modified and from there malignancy arises (Mehta, 1995).
Carcinogenesis depends on inherited and acquired susceptibility factors, on exposure to initiation factors i.e., exogenous and endogenous factors and on promotion and progression factors (Walaszek et al., 2005). Exogenous factors includes nutritional habits, socio-economic status, lifestyle, physical agents (ionising and non-ionising radiation), chemical compounds and biological agents, growth factors (Pitot and Dragan, 1991; Barrett and Anderson, 1993; Minamoto et al., 2000; Lutz, 2002). Unhealthy lifestyle habits such as: excess alcohol consumption; inhalation of tobacco and related products; the ingestion of certain foods and their contamination by mycotoxins; are responsible for higher incidences of certain types of neoplasias in a number of population groups (Gomes-Carneiro et al., 1997; Witenburger, 1999; Gutierrez and Salsamendi, 2001). Endogenous factors include immune system damage and inflammation caused by uncertain aetiology, genetic makeup, age, endocrine balance and physiological condition (Cohen et al., 1991; Barrett and Anderson, 1993; Dewhirst et al., 2003; Ohshima et al., 2003., Ohshima et al., 2005).

The mechanism by which certain carcinogens and radiation cause carcinogenesis is believed to be mediated by free radicals. It is increasingly proposed that reactive oxygen species (ROS) and reactive nitrogen species (RNS) play a key role in human cancer development (Cerutti, 1994; Routledge et al., 1994) especially as evidence is growing that antioxidants may prevent or delay the onset of some types of cancer (Byers and Perry, 1992; Diplock, 1994). ROS and RNS have been shown to possess many characteristics of carcinogens (Cerutti, 1994). Mutagenesis by ROS/RNS could contribute to the initiation of cancer, in addition to being important in the promotion and progression phases. ROS/RNS Cause structural alterations in DNA, e.g. base pair mutations, rearrangements, deletions, insertions and sequence amplification (von Sonntag, 1987; Dizdaroglu, 1993; Epe, 1993). ROS can produce gross chromosomal alterations in addition to point mutations and thus could be involved in the inactivation or loss of the second wild-type allele of a mutated proto-oncogene or tumour-suppressor gene that can occur during tumour promotion and progression, allowing expression of the mutated phenotype (Cerutti, 1994). ROS/RNS also affect cytoplasmic and nuclear signal transduction pathways (Schreck et al., 1992; Burdon, 1995) and can lead to displacement of the inhibitory subunit from the cytoplasmic transcription factor nuclear factor κB, allowing the activated factor to migrate to the nucleus (Schreck et al., 1992). These free radicals
also modulate the activity of the proteins and genes that respond to stress and which act to regulate the genes that are related to cell proliferation, differentiation and apoptosis (Cerutti, 1994; Sarafan and Bredesen, 1994; Burdon et al., 1995). Damage to DNA by ROS/RNS appears to occur naturally, in that low steady-state levels of base damage products have been detected in nuclear DNA from human cells and tissues (Floyd et al., 1986; Ames, 1989; Musarrat and Wani, 1994).

2.2. Influences of stress on cancer relevant biological processes

Stability in the internal environment of a living organism is the result of a complex equilibrium, which is constantly challenged by intrinsic or extrinsic forces, physical or psychological stimuli, known as stressors, which can endanger the survival of an individual. This tendency towards stability is called homeostasis. The term ‘stress’ although having a very broad meaning, generally describes a state of disturbed homeostasis, harmony and equilibrium (Levine and Ursin, 1991; Weiner, 1992; Johnson et al., 1992). Stress can be classified as physical or psychological: of the two classes, psychological stress appears to be more potent stressor (Johnson et al., 1992). Physical and psychological stressors experienced in combination could further exacerbate the stress response. A growing body of basic research and clinical studies suggest that stress and other psychosocial variables including low social support and chronic social isolation contribute to cancer progression and other disease states.

Psychological distress can negatively influence multiple cancer relevant biological processes. Cancer initiation and progression is a complex set of processes that rely on multiple steps including environmental exposures and behaviours, genetic changes, evasion of apoptosis, proliferation, escape from immune surveillance, vascularization, and metastases. There is emerging evidence that psychosocial stress can influence the course of disease at many points during this process (Antoni et al., 2006b). Psychological distress is associated with health behaviours that may promote tumour growth and development. Distress states are associated with increased body mass index (BMI) and greater waist circumference (Wing et al., 1991). Increased body weight, especially central adiposity, is associated with increased risk for breast cancer (Connolly et al., 2002). Psychological distress states
also relate to gene function. Psychological stress is associated with increased DNA damage and poorer DNA repair (Gidron et al., 2006; Flint et al., 2007) and DNA repair pathways are important in the etiology of breast cancer.

Stress has long been associated with gastric and duodenal ulcers, hypertension, cardiovascular and cerebrovascular diseases, cancer and aging (Cooper, 1984, 1995; Csermely, 1998). Long-term exposure to stress has detrimental effects on several cell functions in many species, including humans (Kovacs et al., 1996). The influence of stress-inducing conditions on cancer development has been subject of several investigators, both at clinical and experimental levels (La Barba, 1970; Pradhan and Prabhati, 1974; Greer et al., 1979; Riley, 1981; Justice, 1985; Fox, 1995; Croyle, 1998). Stress has been shown to markedly influence incidence, growth, and metastasis and rejection of chemically induced or implanted tumors (Amkraut & Solomon, 1972; Dechambre and Gosse, 1973; Benchfield et al., 1978; Goldman and Vogel, 1984; Lacconi et al., 2000). However, results are contradictory in that both exacerbation and attenuation of tumor development has been reported by stress. Animal experiments have generally documented a positive association between the two variables (Sklar and Anisman, 1979; Sklar and Anisman, 1980; Steplewski et al., 1985; Matsukawa et al., 1997), although some studies have led to opposite conclusions (Amkraut and Solomon, 1972; Pradhan and Prabhati, 1974; Justice, 1985). For example, foot shock enhanced the growth of transplanted neoplastic cells in mice (Sklar and Anisman, 1979) and a similar pattern of results have been reported when psychosocial stress (isolation) was used in animals bearing grafted tumors (Dechambre and Gosse, 1973; Sklar & Anisman, 1980). Tumor rejection of rats receiving Walker 256 sarcoma and inescapable electric shock was lower than that seen in unstressed controls (Visintainer et al., 1982). Perhaps more interesting are reports indicating a beneficial effect of stress on tumor development. The size of virally induced sarcomas in mice was reduced when stress was elicited prior to virus inoculation, while an enhancing effect on tumor growth was seen when stress followed exposure to the virus (Amkraut and Solomon, 1972). In addition, significant inhibition in the growth and development of DMBA-induced mammary tumors was found by exposure of the animals to immobilization, electric shock and an overcrowding-sound situation (Pradhan and Ray, 1974; Bhattacharya and Pradhan, 1979; Ray and Pradhan, 1979). Animals exposed to electroconvulsive
shock and cold showed better rejection and reduced growth of murine sarcoma and lymphoma tumors (Amkraut and Solomon, 1972; Benchfield, 1978). Among many factors, the intensity and duration of stress seem to play a major role. In experiments on tumor induction by DMBA, it was found that animals stressed after the injection of carcinogen had protective effect of stress whereas no protection was observed if stress began before first injection (Steplewski et al., 1985).

It has also been considered that the ability of the organism to cope with stress is critical in determining its overall effect. Consistent with this proposition, escapable foot shock had no effect on the growth of transplanted tumors in mice, while inescapable shock increased tumor size under the same experimental conditions (Sklar and Anisman, 1979). Furthermore, the effect of inescapable shock was mitigated if mice received long-term shock treatment, suggesting a type of adaptation to stress (Sklar and Anisman, 1979). The nature of the relationship between stress associated conditions and cancer has also been investigated in humans. However much attention has been paid to the possible influence of stress on prognosis in patients who already have cancer (Greer et al., 1979; Fox, 1981; Cassileth et al., 1985), while very limited information is available on any possible direct role of stress in the development of neoplasia, at any step (Fox, 1995).

Various mechanisms have been considered as possible mediators of the effect of stress on neoplastic process, which include alteration in the immune and/or neuroendocrine system and in the antioxidant defense status (Boyd et al., 1981; Steplewski et al., 1985; Banu et al., 1988; Wakikawa et al., 1997; Harada et al., 1997; Muqbil and Naheed, 2006). It shown that restraint stress can accelerate the onset of tumour formation and metastasis in female BALB/c mice treated intragastrically with DMBA (Melanie et al., 2010). Altered antioxidant status indicates production of reactive oxygen species such as peroxides, hydroxyl and superoxide anion radicals. There is accumulating evidence to indicate that stress can stimulate numerous pathways leading to an increased production of free radicals (Kovacs et al., 1996; Liu et al., 1996; Liu and Mori, 1999; Matsumoto et al., 1999; Olivenza et al., 2000; Zaidi et al., 2002; Muqbil and Naheed, 2006). It is well known that free radicals generate a cascade, producing lipid peroxidation, protein oxidation, DNA damage and cell death thus contribute to the occurrence of pathological conditions (Kovacs et al., 1996; Liu et al., 1996; Liu & Mori, 1999).
2.2.1. Stress and oxidative stress

Currently, psychological distress has attracted attention with regards to its significant negative effects in treating various diseases, especially cancer (Andersen et al., 1998, Iribarren, 2005; Liu and Wang, 2005; Bultz and Carlson, 2005). There are a variety of stress induced hemodynamic changes that inhibit cellular immune responses and are relevant to cancer prognosis, e.g. NK cytotoxicity and T-cell responses (Andersen et al., 1998; Sieber et al., 1992; Kelly, 1999). The stressors can also induce myocardial ischemia and ventricular arrhythmias in patients with coronary artery diseases (Kawachi et al., 1996; Sant'anna et al., 2003) which associate with oxidative stress. It is thus suggested that psychological stress is associated with increased oxidant production and oxidative damage, and thus long-term exposure to psychological stressors may enhance the risk of many diseases (Kelly, 1999; Liu and Mori, 1999). Several animal stress models of various stressors such as immobilization, chronic unpredictable stress, burn shock and cold-restraint have been developed, and all cause oxidative lipid, protein and DNA damages in tissues (Yoshikawa et al., 1987; Liu et al., 1996; Kovacs et al., 1996; Muqbil and Naheed, 2006; Hasan et al., 2010). Based on the known pathophysiological effects of stress, it is plausible that associated increases in metabolism may lead to increased oxidant production and oxidative damage to cellular macromolecules. Chronic exposure to stress alters the prooxidant-antioxidant balance, which might lead to the development of various human pathological states (Stojilkovic et al., 2005). A single short-term emotional pain stress produced a transient increase of the lipid peroxidation level (Taranova et al., 1994). Marked changes were observed in erythrocyte antioxidant enzymes SOD, CAT and GR and lipid peroxidation levels of rats exposed to acute, repeated and chronic restraint stress (Sahin et al., 2004). Moreover, different stress models are found to have different degrees of influences on enzymatic and non-enzymatic defense systems, protein oxidation and lipid peroxidation (Sahin and Gumuslu, 2004). Emotional stress induced by 24 h immobilization causes TBARS increase in the brain, liver and heart in Wistar rats (Sosnovskii et al., 1992; Sosnovskii and Kozlov, 1992). Stress causes an increase in TBARS and protein carbonyl content and a decrease in the glutathione content suggesting stress does cause universal oxidative damage. Rats subjected to 30 min cold-immobilization stress show increased TBARS and conjugated dienes in the...
liver, heart and stomach (Kovacs et al., 1996). The potentiation of lipid peroxidation by stress may be due to insufficiency of the protective systems (Aydin et al., 2005) as depletion of antioxidants and antioxidant enzymes by stress have been observed in different tissues of rats (Al-Qirim et al., 2002; Zaidi et al., 2005). To understand the role of antioxidant enzymatic defenses in ROS injury following immobilization stress, the effect on mRNA expression of antioxidant enzymes was examined. The mRNA levels of all antioxidant enzymes were markedly decreased in the liver, while no effect was observed in heart, lung and kidney (Oishi and Machida, 2002). Mice subjected to chronic unpredictable stress show increased TBARS and a decrease in the activities of antioxidant enzymes indicating oxidative stress in heart and brain. (Hasan et al., 2010). Antioxidant defense status in peripheral tissues is thus influenced by immobilization and chronic unpredictable stress and tissue specific regulation mechanisms of antioxidants exist in rats and mice.

2.2.2. Stress and DNA damage and repair

One basic trigger of various diseases including cancer, heart disease and neurodegenerative diseases is damage to DNA integrity (Hoeijmakers, 2001). Indeed, DNA damage induced by oxidative stress and deficient DNA-repair may have etiological and prognostic roles in cancer (Musarrat et al., 1996), diabetes and atherosclerosis (Wu et al., 2004). Several studies have examined the effect of stress on DNA integrity as stress has been found to cause production of ROS resulting in oxidative stress and increased lipid peroxidation. Greater production of ROS has been shown to produce increases in oxidative DNA-damage (Kang, 2002), which is an important etiological and prognostic factor in various chronic illnesses (Wu et al., 2004). The mechanisms that would account in part for the relationship between stress and tumor development may be damage to DNA or DNA repair (Glaser et al., 1985). The levels of methyltransferase, a DNA repair enzyme induced in response to carcinogen alkylation damage, were significantly lower in spleens from stressed animals (Glaser et al., 1985). The studies conducted with animals showed that when stress was primarily repeated and not endured on a single occasion alone, it led to DNA-damage. The study by (Fischman et al., 1996) also demonstrates the generality of the effects of stress across stressors. The study by (Irie et al., 2000) demonstrated that DNA-damage may be elicited by non-toxic materials via classical conditioning.
Finally, the findings of (Bagchi et al., 1999) provide evidence that stress-induced DNA fragmentation may be reduced by protective agents, and this may have important clinical implications. Several studies found relations between certain psychological outcomes such as anxiety–tension, depression and anger with DNA-damage (Dimitroglou et al., 2003; Irie et al., 2001a, 2002, 2003, 2005). In human studies (Glaser et al., 1985) assessed the differences in DNA repair in lymphocytes and showed that lymphocytes from highly stressed humans had significantly poorer DNA repair when exposed to X-irradiation than those from lowly stressed subjects. The biomarker of oxidative damage to DNA, 8-OH-dG, is elevated in the liver nuclear DNA of rats subjected to psychological stress (Adachi et al., 1993). These data provide evidence for a direct pathway through which stress could play a role in the incidence of cancer.

2.3. Chemical Carcinogenesis

People are continuously exposed exogenously to varying amounts of chemicals that have been shown to have carcinogenic or mutagenic properties in experimental systems. Exposure can occur exogenously when these agents are present in food, air or water, and also endogenously when they are products of metabolism or pathophysiologic states such as inflammation. It has been estimated that exposure to environmental chemical carcinogens may contribute significantly to the causation of a sizable fraction, perhaps a majority, of human cancers, when exposures are related to life style factors such as diet, tobacco use etc. Chemically induced neoplasia is a multistep process involving DNA damage and cell proliferation. Chemical carcinogens impact on various stages of this process and function through modification of cellular and molecular events. On the basis of the apparent differences by which chemicals participate in the carcinogenic process, they may be defined as genotoxic or epigenetic (non-genotoxic) (Williams and Weisburger, 1983). Genotoxic agents usually refer to chemicals that directly damage genomic DNA, which in turn can result in mutation and/or clastogenic changes. Such chemicals are frequently activated in the target cell and produce a dose-dependent increase in neoplasm formation (Pitot et al., 1981). In contrast, non-genotoxic compounds appear to function through non-DNA reactive or indirect DNA reactive mechanisms. Although much less is known about the exact mode of action of non-
genotoxic carcinogens, they modulate cell growth and cell death. Changes in gene expression and cell growth parameters are paramount in the action of nongenotoxic carcinogens. These agents frequently function during the promotion stage of the cancer process (Kolaja and Klaunig, 1996). Two possible mechanisms have been proposed for the induction of cancer. In one, an increase in DNA synthesis and mitosis by a nongenotoxic carcinogen may induce mutations in dividing cells through misrepair. With continual cell division, mutations will result in an initiated preneoplastic cell that may clonally expand to a neoplasm. In addition, nongenotoxic agents may serve to stimulate the selective clonal growth of already spontaneously initiated cells (Ames and Gold, 1990). In maintaining cell number within a tissue, equilibrium exists between cell proliferation and cell death. The cancer process thus is a result of an imbalance between cell growth and death. Experimental evidence supports an important role for reactive oxygen species in the cancer process. Increases in the reactive oxygen in the cell, through either physiological modification or through chemical carcinogen exposure, contribute to the carcinogenic processes. This may be via genotoxic effects resulting in oxidative DNA adducts or through modification of gene expression (Klaunig and Kamendulis, 2004). There are plethoras of carcinogens, of which few are described below. Although oxidative stress is described herein as a unifying thread, other modes of action may also be involved. ROS are commonly generated by electron transfer, although non-electron transfer routes can also occur, e.g., Radiation. The activity of peroxides and radiation provide compelling evidence for the important participation of oxidative stress.

2.3.1. Radiation

The specific types and levels of radiation that cause cancer remain a matter of speculation (Doll, 1998; Jaworowski, 1999; Marks, 1999). Each day, over a billion particles of radiation cross paths with a human body. In spite of the disputation, there is a strong correlation between radiation exposure (generally, wavelengths under 320 nm) and cancer (Fry and Ley, 1984). Radiation damage to DNA may come about either directly or indirectly. Non-melanoma skin cancers are the most common, and are thought to be brought on by excessive exposure to UV rays (Sarasin, 1999). Those with xeroderma pigmentosum syndrome – a rare disease wherein the skin contains very little repair or defense mechanisms - have very high
skin cancer incidences. Hence, DNA repair and defense are important in the promotion and progression of skin cancer. High-energy radiation, e.g., x-rays, gamma rays, and particle radiation, affect matter by breaking molecular bonds or ejecting electrons (ionization). When ionizing radiation impinges upon an aqueous environment, O-H bonds within water molecules break to form the highly reactive •OH moieties (Plumb et al., 1999). •OH insults DNA primarily by abstracting H• at various places on the helix. This process leaves behind radical lesions, which can form various oxyl and peroxyl radicals by reacting with O₂ or superoxide radical, the resultant peroxides can undergo Fenton type reactions (Rashid et al., 1999). Malonaldehyde, formed by oxidative degradation of the ribose portion of DNA, is a chief product in such a sequence, in addition to 8-OH dG. Since the proximate nuclear environment is aqueous, at low to average levels of radical scavengers, •OH induced DNA damage is the predominant type caused by ionizing radiation (Plumb et al., 1999). The mechanistic picture is complicated by generation of cationic-type species that alkylate cellular constituents (Seifter et al., 1984).

2.3.2. Peroxides

Various peroxides have long been associated with carcinogenesis (Hix and Augusto, 1999). More than forty years ago, hydrogen peroxide was reported to be an initiator (Kovacic, 1959). In fact, many carcinogens indirectly generate H₂O₂, which in turn brings about increased amounts of ROS (Kovacic, 1959). More recent experimentation has elucidated many specifics concerning H₂O₂-induced DNA damage (Korzets et al., 1999), including its role in tumor promotion, oncogene activation, and gap-junction disruption (Huang et al., 1999). Hydrogen peroxide has also been implicated in apoptosis by attacking regulatory proteins (Riou et al., 1999). The cytotoxicity and tumor-promoting ability of benzoyl peroxide (BPO) have also been dealt with (Gopalakrishna et al., 1999). BPO is involved in the oxidation of several key enzymes, including protein kinases, which may play an important mechanistic role in promotion. Tumors may be caused by tert-Butyl hydroperoxide by forming DNA adducts via reactive methyl radicals (•CH₃) (Hix and Augusto, 1999). Transition metal ions attached to the DNA are apparently involved in the radical sequence. Enzymatic and non-enzymatic cleavage of peroxides generates three predominant types of radicals: alkoxy, peroxy, and
2.3.3. Haloalkanes (HAs)

Carbon tetrachloride (CCl₄) and other haloalkanes (HAs) are widely used industrial solvents. The carcinogenicity and toxicity associated with CCl₄ exposure is well established (Frezza et al., 1994; Brennan and Schiestl, 1998). Many HAs, including CCl₄, are procarcinogens that become activated via a sequence of redox reactions whereby CYP450 acts as the principal catalyst in the dehalogenation (Yao et al., 1994). In a study involving ¹⁸O, several oxidative paths and oxidative species were verified and the isotope was found to be present in large amounts in lipids and other macromolecules, indicating oxidation (Hatch et al., 1988).

\[
\text{CCl}_4 \rightarrow \text{CCl}_4^- \rightarrow \text{Cl}_3\text{C} \rightarrow \text{Cl}_3\text{COO}^- \rightarrow \text{Oxy Radicals}
\]

Metabolism of CCl₄

Most carcinogens or their metabolites appear to interact directly with DNA as an important component in the oncogenic sequence. The radicals generated, during metabolism, in the immediate vicinity of the nucleic acids then effect chain scission. HA activity is marked by an ability to penetrate biological membranes (Ludek et al., 1998). Research involving HAs dealt with their effects on enzyme signaling (Roghani et al., 1987), tissue repair proteins and oncogenes (Camandola et al., 1999), including induction of human proto-oncogenes in transgenic mice (Tsunematsu et al., 1994). Studies with CHCl₃ (Brennan and Schiestl, 1998) and CBr₄ (DeGroot & Noll, 1989) indicate activity resembling that of CCl₄ while BrCH₂CH₂Br and CCl₄ were found to be synergistic (Camandola et al., 1999).

2.3.4. Aromatic amines

(i) Hydrocarbon Types

A detailed review incorporates a large body of knowledge concerning aromatic amines and their link to cancer (Vineis and Pirastu, 1997). Human exposure is primarily through various dyes, rubber, and oil industries, agricultural settings, and tobacco smoke. There are numerous case studies linking naphthylamine and benzidine to bladder cancer. It is proposed that bladder cancer in smokers comes...
about primarily through exposure to ArAs, not PAH or other smoke byproducts. Twenty carcinogenic ArAs and ten noncarcinogenic ones were analyzed using real-time, organ specific testing (Sasaki et al., 1999). Of the twenty, aniline, benzidine, many of their derivatives and other aromatic amines damaged DNA. Nine of the ten non-carcinogenic amines exhibited no adverse effects. Ring size of aromatic amines is directly related to their potency (Shapiro et al., 1998). Larger ring adducts intercalate and bind DNA grooves, which can distort the helical shape and inhibit repair. Some studies also suggest a role for ROS in aromatic amines carcinogenicity (Loft et al., 1999).

(ii) Heterocyclic Types (Cooked Foods)

Pyrolysis of food can create several types of heterocyclic amines, many of which are suspected in carcinogenesis (Adamson et al., 1996; Schut and Synderwine, 1999). They are generated primarily by reactions of amino acids in meats, and the amount produced depends upon the duration and temperature of cooking (Zheng et al., 1998; Skog et al., 1998). Carcinogenic mechanisms involve ring epoxidation to form a phenol, or a multi-step process beginning with N-hydroxylation by CYP450 to create the RNHOH moiety which is esterified to RNHOCOR by N-acetyl transferase (NAT) (Manson and Benford, 1999). The two most prevalent heterocyclic amines in cooked foods are the imidazoquinoxaline (Ryu et al., 1999) and the imidazopyridine (Nagao et al., 1998). Exposure to former causes DNA damage and overexpression of oncogenes (Ryu et al., 1999), and later induces guanine specific DNA adducts (Totsuka et al., 1996).

2.3.5. Aromatic hydrocarbons

Exposure to monocyclic (Wiemels et al., 1999) and polycyclic aromatic hydrocarbons (PAHs) is intimately linked to cancer incidence (Boffetta et al., 1997). The chemicals are commonly found in industrial settings, diesel exhaust, foods and cigarette smoke (Schoket, 1999). Monocyclic and PAHs are procarcinogens; various endogenous entities, including CYP450, oxidize the rings to the active forms which are implicated in DNA binding and oxidative stress. PAH potency rises as the number of aromatic rings increases (Till et al., 1999), whereby the larger adducts may interfere to a greater extent with various repair processes. Dibenzopyrene (DBP), a six-ringed aromatic hydrocarbon, is a more potent carcinogen than
dimethylanthracene (3 rings) or benzopyrene (5 rings) (Arif et al., 1999). Several endogenous moieties can be involved as catalysts in their metabolism, including CYP450, which can oxidize the rings to various epoxides. Since the repeated application of the carcinogenic PAHs alone results in production of tumors, many of which are malignant carcinomas (Shubik, 1950), they are considered complete carcinogens (Berenblum, 1974). Complete carcinogens carry out both initiating and promoting functions. When used in both initiating and promoting stages, they yield a higher incidence of carcinomas than does initiation with a PAH and promotion by TPA (Shubik, 1950). It was suggested that malignant tumor formation results from two or more carcinogen-induced mutations and that the role of promotion is to enlarge the size of the target cell population available for the second mutation (Hennings et al., 1983). Papillomas induced by repeated carcinogen application arise from significantly more cells than those induced by the carcinogen promoter sequence (Reddy and Fialkow, 1983)

2.3.5.1. Metabolic activation of PAHs

PAHs are ubiquitously distributed carcinogens in the environment and their carcinogenic potentials have been extensively studied in experimental animal models (Conney, 1982). PAHs acquire carcinogenecity only after they have been activated by xenobiotic-metabolizing enzymes (Phase I enzymes) to highly reactive metabolites capable of attacking cellular DNA. Cytochrome P450 (CYP) enzymes are central to the metabolic activation of these PAHs to epoxide intermediates, which are converted with the aid of epoxide hydrolase to the ultimate carcinogens, diol epoxides (Shimada & Kuriyama, 2004). Historically, CYP1A1 had been thought to be the sole enzyme responsible for the metabolic activation of most of the carcinogenic PAHs to reactive electrophiles in mice, rats, and rabbits (Conney, 1982). However, recent studies have established that CYP1B1 also activates PAHs to reactive metabolites at rates similar to or even higher than CYP1A1 in experimental animals and humans. As a further cause of concern, human CYP1B1 has also been shown to metabolize 17β-estradiol to a 4-hydroxylated product, a chemical considered to cause breast cancer in women (Spink et al., 1998). Both CYP1A1 and 1B1 are expressed mainly in extrahepatic organs and thus make a major contribution to the incidence of cancers in these organs, when PAHs and other carcinogens are ingested into an animal’s body (Shimada et al., 1996). PAHs induce
several xenobiotic metabolizing enzymes, including CYP1A1 and 1B1, through the aryl hydrocarbon receptor. Many studies have demonstrated that most carcinogenic PAHs are activated by the combined actions of CYPs and epoxide hydrolases to highly reactive diol-epoxides that initiate cell transformation (Gelboin, 1980; Conney, 1982). For a number of PAHs, including 7,12-dimethylbenz(a)anthracene (DMBA) the ultimate carcinogen is a so-called bay-region dihydrodiol epoxide produced during cellular metabolism (Kapitulnik et al., 1978; Slaga et al., 1984).

Biological systems have developed methods for dealing with electrophilic xenobiotics produced during Phase-I metabolisms; they are generally classified as Phase-II enzymes. Many antioxidants and anticarcinogenic compounds that block the toxic and neoplastic effects of carcinogen share in common the ability to elevate levels of Phase-II detoxification enzymes, i.e., glutathione-S-transferases (GSTs), quinone reductase, and UDP-glucuronosyltransferases (Wattenberg, 1992; Hanausek et al., 2003; Walaszek et al., 2004). Substantial evidences have accumulated to suggest that induction of Phase II enzymes is a causal mechanism for protection since these enzymes divert ultimate carcinogens from reacting with critical cellular macromolecules (Prochaska et al., 1992).

2.4. N-Nitrosamines

Nitrosamines are compounds having the general structure:

\[
\begin{align*}
\text{R}_1 & \text{N} - \text{N} = \text{O} \\
\text{R}_2 & \\
\end{align*}
\]

where R1 and R2 are alkyl or aryl groups. Nitrosamines are present in water, soil and air. They can be found contaminating food, feeding stuff (where they create the highest risk for health), drugs, cosmetics, and pesticides (Hecht, 1997; Hecht and Hoffman, 1988; Osterdahl, 1990). Nitrosamines are absorbed by skin, airways and the alimentary tract (Low, 1974). There is evidence that nitroso compounds may be generated in vivo from nitrites or nitrates and primary, secondary and tertiary amines in organs of people who apparently were not exposed to these compounds (Brendler et al., 1992; Hinuma et al., 1990).
Human exposure to a wide range of N-nitrosocompounds occurs from diet, tobacco smoking, work place and drinking water (Bartsch and Spiegelhalder, 1996; Tricker and Preussmann, 1991) which are the major source of exposure in the general population (Tricker, 1997). Nitrate and nitrite are added to meat and fish for the purpose of preservation, as colour fixatives and as flavouring. Ingestion of nitrite and nitrate can result in the endogenous formation of nitroso compounds, particularly in the presence of nitrosatable precursors, such as primary amines, in the acidic condition of the stomach (Lin et al., 2002). Preformed exogenous nitrosamines are found mainly in cured meat products, smoked preserved foods, foods subjected to drying by additives such as malt in the production of beer and Whiskey, pickled and salty preserved foods (Tricker and Preussmann, 1991). Available data suggest that nitrosamines are found more frequently and at higher concentration in Asian foods than in Western foods (Hotchkiss, 1989). Nitrosamines are comparatively stable under conditions present in organisms, before being subjected to degradation to biologically active derivatives (Przezdziecki, 1980; Lake et al., 1982; Huang et al., 1992;). Nitrosamines reach the liver by the blood stream. In the liver microsomes, enzymes are present which are responsible for reactions of the first and second phase of biotransformation of nitrosocompounds. In the first phase of biotransformation of nitrosamines, hydroxylation and dealkylation are the main reactions. In the second phase, the products of the first phase undergo transformation to polar metabolites by the action of specific enzymes which conjugate them with glucuronic or sulfuric acids or aminoacids or glutathione. Enzymes of the first and second phases of biotransformations were detected not only in liver but also in the intestine, kidney, lungs, brain, skin and placenta (Przezdziecki, 1980; Hinuma et al. 1990;). Biotransformation reactions are catalyzed by microsomal enzymes dependent on cytochrome P-450, i.e. a set of hemoproteins catalyzing the activation of molecular oxygen and transfer of oxygen to lipophilic molecule of the xenobiotic (Hanke, 1980; Labuc and Archer, 1982; Lutz, 1984; Hiroshi et al., 1992). Nitrosamines are excreted partially in urine and exhaled in air. Remaining nitrosamines are degraded to carbon dioxide by active intermediates (Nikonorow and Urbanek, 1987). Active intermediates are very important from the toxicological point of view as many of them have carcinogenic activity.
2.5. N-Nitrosodiethylamine (NDEA)

N-Nitrosodiethylamine (NDEA), also known as diethyl nitrosamine or DEN, is a slightly yellow liquid with a boiling point of 175-177°C. It is soluble in water, ethanol, diethyl ether and organic solvents.

Its chemical structure is

\[
\begin{align*}
\text{CH}_3 & \quad \text{CH}_2 \\
\text{N} & \quad \text{N} \equiv \text{O} \\
\text{CH}_3 & \quad \text{CH}_2
\end{align*}
\]

NDEA has had extensive use as an experimental carcinogen. NDEA has been found in a variety of products that would result in human exposure, including mainstream and sidestream tobacco smoke, meat, whiskey, cured meats, salami, millet flour and dried cuttlefish (Brunnemann and Hoffmann, 1978; Hoffmann et al., 1980; Sen et al., 1980; Loepky, 1999). The International Agency for Research on Cancer review (IARC 1978, 1982, 1987) concluded that NDEA was carcinogenic in all animal species tested. There are many NDEA bioassays and target organ studies in a variety of animal species and strains, including a large dose/response study in rats by the British Industrial Biological Research Association (BIBRA).

NDEA is metabolized primarily in the liver by cytochrome P450 to ethylacetoxyethyl-nitrosamine. This intermediate can be conjugated by the phase II enzymes to a non-toxic compound or it can produce ethyl diazonium ion that directly ethylates cellular macromolecules (Muzio et al., 1999). Metabolic activation of NDEA by cytochrome P450 enzymes is responsible for its cytotoxic, mutagenic and carcinogenic effects. Instrumental to them is the increased in ROS production caused by NDEA (Bansal et al., 2000). N-Nitrosodiethylamine has been suggested to cause oxidative stress and cellular injury due to the enhanced formation of free radicals (Ramakrishnan et al., 2006; Valko et al., 2006). NDEA has been shown to be metabolized to its active ethyl radical metabolite, and the reactive product interacts with DNA causing mutation which would lead to carcinogenesis (Anis et al., 2001).

The putative mechanism of NDEA-DNA adduct formation is shown in Fig. 2. DNA-adduct formation proceeds through an ethyl diazonium ion intermediate and
evolution of N₂ (Michejda et al., 1982; Singer and Grunberger, 1983). Since this is a relatively simple and rapid alkylation mechanism, NDEA may produce DNA adducts in any tissue with appropriate activating P450 isozymes. Nitrosamine alkylation in each organ reflects the local level of bioactivation capacity, since the diazonium ion is too reactive to be transported to other organs in significant amounts. Additionally, P450 isozymes are responsible for detoxification of NDEA, thereby adding to the complexity of the biotransformation. After NDEA is bioactivated to an electrophilic ethyl diazonium ion, it undergoes reaction with nucleophiles, including DNA bases, to form adducts. (Saenger, 1984).

![Diagram of NDEA biotransformation](image)

**Fig. 2. Biotransformation of NDEA and mechanism of DNA-formation.**

### 2.6. Oxidative stress and reactive oxygen species

Oxidative stress occurs in a cell or tissue when the concentration of reactive oxygen species (ROS) generated exceeds the antioxidant capability of that cell (Sies, 1991). ROS can be produced both endogenously and exogenously (Figure 3). Endogenous oxidative stress can be the result of normal cellular metabolism and oxidative phosphorylation. The metabolism of substances by the P450 enzyme system
generates oxygen free radicals through normal or futile cycling mechanisms (Parke and Ioannides, 1990). Exogenous sources of ROS can also impact on the overall oxidative status of a cell. Drugs, hormones, and other xenobiotic chemicals can produce ROS by either direct or indirect mechanisms (Trush and Kensler, 1991; Halliwell, 1996). Alternatively, oxidative stress can also occur when there is a decrease in the antioxidant capacity of a cell. Non enzymatic antioxidant levels (vitamin E, vitamin C, glutathione, etc.) and enzymatic antioxidant levels (superoxide dismutase, glutathione peroxidase, and catalase) in the cell can be decreased through modification in gene expression, decreased in their uptake in the diet, or can be overloaded in ROS production, which creates a net increase in the amount of oxygen free radicals present in the cell (Vuillaume, 1987; Barber and Harris, 1994). Several human chronic disease states including cancer have been associated with oxidative stress produced through either an increased free radical generation and/or a decreased antioxidant level in the target cells and tissues (Trush and Kensler, 1991; Rice-Evans and Burdon, 1993). A role for reactive oxygen radicals in the etiology of cancer is supported by epidemiologic studies. Specifically these epidemiologic studies illustrated the protective role for antioxidants against cancer development (Ames, 1983; Willett and MacMahon, 1984) and a correlation between tumour induction and the intake of high concentrations of transition metals such as iron, which facilitate the production of free radicals (Nelson, 1992; Stevens and Nerishi, 1992). The formation of oxidative stress may result in damage to critical cellular macromolecules including DNA, lipids, and proteins. Oxidative DNA damage may participate in ROS-induced carcinogenesis (Breimer, 1990). A common form of damage is the formation of hydroxylated bases of DNA, which are considered an important event in chemical carcinogenesis (Breimer, 1990; Chaudhary et al., 1994). This adduct formation interferes with normal cell growth by causing genetic mutations and altering normal gene transcription. Several different pathways by which oxidative DNA damage leads to mutations have been proposed, including chemical modification of nucleotide moieties in DNA causing alteration in their hydrogen bonding, exacerbation of polymerase-specific hot spots, conformational change in the DNA templates, and the induction of a DNA polymerase conformation that is error prone (Feig et al., 1994). Formation of 8-hydroxy-2'-deoxyguanosine (8-OHdG) [an oxidative modification of DNA produced
by hydroxylation in the C-8 position of deoxyguanosine residues by the hydroxyl radical (Floyd, 1990) has been used as a measurement of oxidative DNA damage.

A substantial body of evidence has been produced that links the production of reactive oxygen radicals, and subsequent oxidative stress and damage, to the pathogenesis of age-related and chronic diseases including cancer (Vuillama, 1987; Trush and Kensler, 1991; Witz, 1991; Guyton & Kensler, 1993). Reactive oxygen species (ROS) are derived from the metabolism of molecular oxygen (Halliwell, 1999). Molecular oxygen (dioxygen) has a unique electronic configuration and is itself a radical. The addition of one electron to dioxygen forms the superoxide anion radical ($\text{O}_2^-$) (Miller et al., 1990). Superoxide anion, arising either through metabolic processes or following oxygen "activation" by physical irradiation, is considered the "primary" ROS, and can further interact with other molecules to generate "secondary" ROS, either directly or prevalently through enzyme-or metal catalyzed processes (Valko et al., 2005). These include singlet oxygen ($^1\text{O}_2$), hydrogen peroxide ($\text{H}_2\text{O}_2$), and the highly reactive hydroxyl radical ($\text{OH}$). The deleterious effects of oxygen are said to result from its metabolic reduction to these highly reactive and toxic species (Buechter, 1988). ROS normally exist in all aerobic cells in balance with biochemical antioxidants. Oxidative stress occurs when this critical balance is disrupted because of excess ROS, antioxidant depletion, or both (Scandalios, 2002). To counteract the oxidant effects and to restore redox balance, cells must reset important homeostatic parameters. ROS are not always harmful metabolic byproducts; when tightly regulated, ROS can act as intracellular signaling molecules (Klein & Ackerman, 2003). ROS can be produced by both endogenous and exogenous sources. In living cells, the major sources of endogenous ROS are hydrogen peroxide and superoxide anion, which are generated as by products of cellular metabolism such as mitochondrial respiration (Nohl et al., 2003). Alternatively, hydrogen peroxide may be converted into water by the enzymes catalase or glutathione peroxidase. Variability or inductive changes in the expression of these enzymes can significantly influence cellular redox potential. ROS can cause tissue damage by reacting with lipids in cellular membranes, nucleotides in DNA (Ahsan et al., 2003), sulphhydryl groups in proteins (Knight, 1995) and cross-linking/fragmentation of ribonucleoproteins (Waris & Alam, 1998). The relatively unreactive superoxide anion radical is converted by superoxide
dismutase (SOD) into H₂O₂, which in turn take part in the "Fenton reaction", with transition metal ion (copper or iron) as catalysts, to produce the very reactive hydroxyl radical (Aruoma et al., 1989; Halliwell & Gutteridge, 1990; Halliwell and Gutteridge, 1992; Halliwell, 1993). ROS can be produced by a host of exogenous processes. Environmental agents including nongenotoxic carcinogens can directly generate or indirectly induce ROS in cells (Rice-Evans and Burdon, 1993). The induction of oxidative stress and damage has been observed following exposure to xenobiotics of varied structure and activities (Klaunig et al., 1997).

![Endogenous Sources](image1.png)

**Fig. 3.** Pathways illustrating the sources of reactive oxygen species and its role in the development of cancer.

**2.6.1. Generation of ROS:**

During the earliest periods of life on earth, living organisms were chiefly anaerobic (Sahnoun et al., 1997). As evolution took place in the presence of steadily increasing
amounts of atmospheric oxygen, life forms adopted numerous mechanisms to
defend against, as well as utilize, the reactivity of the molecule. The marked activity
can be attributed to the propensity of molecular oxygen to form various radical
species. The formation of ROS is guided by external and internal agents, e.g.,
irradiation, exogenous chemicals, and endogenous entities, e.g., phagocytes and
enzymes, such as cytochrome P450 mono-oxygenases (CYP450). Likewise, creation
of ROS can be ebbed or reversed in the presence of certain agents, e.g., antioxidants
and various enzymes, including superoxide dismutase (SOD), glutathione reductase
(GR) and catalase (CAT). Aerobic life, as we know it, might be viewed as a contest
between the formation and deactivation of ROS. In this light, oxidative stress can be
defined as a state wherein this process becomes unbalanced. CYP 450, a chief
enzyme in many oxidative processes, manipulates molecular oxygen, with the help
of a reducing agent (designated RH2 in reaction 1), in order to monoxygenate a
targeted substrate (Halliwell and Gutteridge, 1999). Usually, the oxidation is helpful
to the organism, e.g., hydroxylation of Phenobarbital, leading to detoxification and
excretion. However, as in the case of aromatic hydrocarbons, carbon tetrachloride
and others, CYP 450 oxidative metabolites can be harmful.

$$\text{Substrate-H} + \text{O}_2 + \text{RH}_2 \rightarrow \text{Substrate-OH} + \text{R} + \text{H}_2\text{O} \quad (1)$$

_Mono-oxygenation of substrate by cytochrome P450_

Molecular oxygen often undergoes a single electron reduction to form the
superoxide radical anion (SO$^-$) (Hauptmann and Cadenas, 1997). The production of
superoxide occurs mostly within the mitochondria of a cell (Cadenas and Sies.
1998). The mitochondrial electron transport chain is the main source of ATP in the
mammalian cell and thus is essential for life. During energy transduction a small
number of electrons “leak” to oxygen prematurely, forming the oxygen free radical
superoxide (Valko, 2004; Kovacic, 2005). _In vivo_ SO$^-$ can be converted to various
oxidative species, including: peroxides and various oxy radicals, namely hydroxyl
(‘OH), peroxyl (ROO$^\cdot$) alkoxyl (RO$^\cdot$). Such entities, including SO$^-$ itself, can cause
several internal anomalies, including: enzyme inactivation, lipid and protein
peroxidation, and DNA oxidation (Halliwell and Gutteridge, 1999). Superoxide
radical is usually neutralized by enzymatic conversion to the less reactive, non-
radical hydrogen peroxide via SOD (reaction II). This activity is often observed occurring along the mitochondrial electron transport chain.

\[
2\text{O}_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \quad (\text{II})
\]

\textit{Dismutation of superoxide}

Hydrogen peroxide, which has various duties and metabolic fates, can be Fenton catalyzed to form one of the most powerful ROS, the hydroxyl radical (reaction III). The Fenton reaction is part of a net transformation called the Haber-Weiss reaction, whereby superoxide and molecular oxygen undergo redox cycling with a catalyst, usually iron. (reaction IV). The redox state of the cell is largely linked to an iron (and copper) redox couple and is maintained within strict physiological limits. It has been suggested that iron regulation ensures that there is no free intracellular iron; however, in vivo, under stress conditions, an excess of superoxide releases “free iron” from iron containing molecules. The release of iron by superoxide has been demonstrated for [4Fe-4S] cluster containing enzymes of the dehydratase-lyase family (Liochev and Fridovich, 1994). The released Fe\(^{2+}\) can participate in the Fenton reaction, generating highly reactive hydroxyl radical. Thus under stress conditions, O\(_2^-\) acts as an oxidant of [4Fe-4S] cluster-containing enzymes and facilitates OH production from H\(_2\)O\(_2\) by making Fe\(^{3+}\) available for the Fenton reaction (Leonard et al., 2004; Valko et al., 2005). Processes like these compel organisms to limit the presence of catalytic entities like metals.

\[
\text{Fe(II)} + \text{H}_2\text{O}_2 \rightarrow \text{Fe(III)} + \text{HO}^- + \text{HO}^- \quad (\text{III})
\]

\textit{Fenton reaction}

\[
\text{Fe (III)} + \text{O}_2^- \rightarrow \text{Fe(II)} + \text{O}_2
\]

\[
\text{Fe (II)} + \text{H}_2\text{O}_2 \rightarrow \text{Fe(III)} + \text{HO}^- + \text{HO}^-
\]

\[
\text{O}_2^- + \text{H}_2\text{O}_2 \rightarrow \text{HO}^- + \text{OH}^- + \text{O}_2 \quad (\text{IV})
\]

\textit{Haber-Weiss reaction}

The hydroxyl radical is the neutral form of the hydroxide ion. The hydroxyl radical has a high reactivity, making it a very dangerous radical with a very short \textit{in vivo} half-life of approx. 10\(^{-9}\)s (Pastor, 2000). Thus when produced \textit{in vivo} OH reacts close to its site of formation.
Catalase keeps the level of hydrogen peroxide in check via reactions that form water and molecular oxygen (reaction V). In addition, glutathione (GSH), which reacts with hydrogen peroxide to form water and oxidized glutathione (GSSG), can act in a variety of ways to combat radical formation (Halliwell and Gutteridge, 1999). Many other species, including metals, metal chelators, cofactors, antioxidants, singlet oxygen, and enzymes are involved in these redox transformations.

\[
2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2
\]

*Decomposition of hydrogen peroxide by catalase*

Additional reactive radicals derived from oxygen that can be formed in living systems are peroxyl radicals (ROO·). The simplest peroxyl radical is HOO·, which is the protonated form (conjugate acid; pKa ~ 4.8) of superoxide and is usually termed either hydroperoxyl radical or perhydroxyl radical. With this pKa value, only ~0.3% of any superoxide present in the cytosol of a typical cell is in the protonated form (De Grey, 2002). It has been demonstrated that hydroperoxyl radical initiates fatty acid peroxidation by two parallel pathways: fatty acid hydroperoxide (LOOH)-independent and LOOH dependent (Aikens and Dix, 1991). The LOOH-dependent pathway of HO_2· initiated fatty acid peroxidation may be relevant to mechanisms of lipid peroxidation initiation *in vivo* (Valko et al., 2007).

### 2.6.2. Oxygen radicals and lipid peroxidation:

Initiation of lipid peroxidation in a membrane or polyunsaturated fatty acid is due to the attack of any species that has sufficient reactivity to abstract hydrogen.

\[
\text{Lipid-H + OH} \rightarrow \text{Lipid + H}_2\text{O}
\]

Since hydrogen atom has only one electron this leaves behind an unpaired electron on the carbon atom. The carbon radical in a polyunsaturated fatty acid tends to be stabilized by a molecular rearrangement to produce a conjugated diene, which rapidly reacts with O_2 to give a hydroperoxy radical.

\[
\text{Lipid} + \text{O}_2 \rightarrow \text{Lipid-O}_2·
\]

(After molecular rearrangement)
Hydroperoxy radicals abstract hydrogen atoms from other lipid molecules – this is the propagation stage of lipid peroxidation and so continues the chain reaction of lipid peroxidation. The hydroperoxy radical combines with the hydrogen atom that it abstracts to give a lipid hydroperoxide R-OOH (Halliwell and Gutteridge, 1984)

\[
\text{Lipid-O}_2^- \text{ + Lipid-H} \longrightarrow \text{Lipid-O}_2^- \text{H + Lipid}
\]

Pure lipid hydroperoxides are fairly stable at physiological temperatures and a major role of transition metals is to catalyze their decomposition. Many metal complexes that can do this are present in vivo. They include simple complexes of iron salts with phosphate ion or phosphate esters such as ADP. Haem, hemoglobin, peroxidase, cytochrome P-450, other cytochromes and non-haem iron proteins are also effective (O’Brien, 1969; Kaschner and Hatefi, 1975; Gutteridge, 1977; Aust and Svingen, 1982). All these should contribute to the propagation of lipid peroxidation in membranes in vivo. A reduced iron compound can react with lipid hydroperoxides (lipid-O2H), in a similar way to its reaction with H2O2 to give alkoxy (lipid-O\textsuperscript{-}) radicals.

\[
\text{Lipid-O}_2\text{H} + \text{Fe}^{2+}\text{-complex} \longrightarrow \text{Fe}^{3+}\text{-complex} + \text{OH}^- + \text{lipid-O}^-
\]

(Fenton reaction)

With an iron (III) compound a peroxy (lipid- O\textsuperscript{2-}) radical will form:

\[
\text{Lipid-O}_2\text{H} + \text{Fe}^{3+}\text{-complex} \longrightarrow \text{lipid- O}_2^- + \text{H}^+ + \text{Fe}^{2+}\text{-complex}
\]

Both alkoxy and peroxy radicals stimulate the chain reaction of lipid peroxidation by abstracting further hydrogen atoms.

2.6.3. Lipid peroxidation and carcinogenesis:

Lipid peroxidation generates a constellation of products among which are reactive electrophiles such as epoxides and aldehydes (Esterbauer, 1985; Janero, 1990). The major aldehyde product of lipid peroxidation other than Malondialdehyde (MDA) (Marnett, 1999) is 4-hydroxy-2-nonenal (HNE) (Valko et al., 2006). MDA is mutagenic in bacterial and mammalian cells and carcinogenic in rats (Mukai and Goldstein, 1976). It is highly electrophilic as well as nucleophilic and reacts not only with cellular nucleophiles but leads to self-condensation to form MDA oligomers.
The dimer of MDA is approximately equipotent to monomeric MDA as a mutagen (Riggins and Marnett, 2001). MDA reacts with nucleic acid bases at physiological pH to form adducts to dG, dA and dC (Fig.4) (Seto et al., 1981; Nair et al., 1984; Marnett et al., 1986; Stone et al., 1990 a,b).  

**Fig 4.** Generation of monomeric adducts from MDA reaction with DNA.

In addition, MDA can attack amino groups on the protein molecule to form both intramolecular cross links and also cross-links between different protein molecules.

Enzymes that require –NH₂ or –SH groups for their activity are usually inhibited during lipid peroxidation e.g., the glucose-6-phosphate enzyme found in liver microsomal fractions is inhibited as its –SH groups are attacked. HNE and other low molecular weight products of lipid peroxidation have been shown to inhibit protein synthesis and to interfere with the growth of bacteria and animal cells in culture.
The production of MDA and its reactions with DNA and proteins provides a link between lipid peroxidation and genetic disease. Furthermore, DNA adduction by MDA correlates to alterations in cell cycle control and gene expression in cultured cells (Ji et al., 1998). Thus lipid peroxidation must be considered significant endogenous source of DNA damage and mutations that contribute to human genetic diseases including cancer.

2.6.4. Oxidative DNA damage and carcinogenesis:

Damage to DNA by ROS has been widely accepted as a major cause of cancer (Ames, 1983). In a given cell, an estimated $10^5$ oxidative lesions per day are formed (Fraga et al., 1990). Over 100 oxidative DNA adducts have been identified (Von Sonntag, 1987; Dizdaroglu, 1992; Demple and Harrison, 1994). ROS can directly produce single- or double-stranded DNA breaks, purine, pyrimidine, or deoxyribose modifications, and DNA cross-links. Persistent DNA damage can result in arrest or induction of transcription, induction of signal transduction pathways, replication errors and genomic instability, all of which are seen in carcinogenesis (Marnett, 2000; Valko et al., 2006). Division of cells with unpaired or misrepaired damage leads to mutations. Human studies support the experimentally based notion of oxidative DNA damage as an important mutagenic and apparently carcinogenic factor (Loft & Poulsen, 1996). The majority of mutations induced by ROS appear to involve modification of guanine, causing G→T transversions (Higinbotham et al., 1992; Demple et al., 1994; Denissenko et al., 1996; Lunec et al., 2002). If it occurs in critical genes such as oncogenes or tumor suppressor genes, initiation/progression can result (Ames et al., 1993). Indeed, these species can act at several steps in multistage carcinogenesis. It is now assumed that ROS are involved in both the initiation and progression of cancer (Moller et al., 1998). Because of the multiplicity of DNA modifications produced by ROS, it has been difficult to establish the frequency and specificity of mutations by individual oxygen radical induced lesions. Some of these modified bases have been found to possess mutagenic properties. Therefore, if not repaired they can lead to carcinogenesis. Studies show that although all the four bases are modified by ROS, mutations are usually related to modification of GC base pairs, while that of AT base pair rarely leads to mutations (Retel et al., 1993). These mutations are usually base pair substitutions, whereas base deletions and insertions are less frequent. In human tumors, G to T
transversions are the most frequent mutations in the p53 suppressor gene (Brash et al., 1991; Hollstein et al., 1991; Harris & Hollstein, 1993). Using single stranded DNA template in a sensitive forward mutation system, various mutations, including tandem double CC→TT substitution have been observed in DNA treated with oxygen free radicals (Reid and Loeb, 1993). Elevated levels of modified bases in cancerous tissue may be due to the production of large amount of H₂O₂, which has found to be characteristic of human tumor cells (Szatrowski & Nathan, 1991; Olinski et al., 1998). Initiation of cancer in humans by ROS is further supported by the presence of oxidative DNA modifications in cancer tissue (Ames et al., 1993; Poulsen et al., 1998). Many forms of reactive oxygen species are capable of forming oxidized bases. The hydroxyl radical in particular has been shown to produce a number of oxidized DNA lesions (Marnett, 2000). The reactivity of the hydroxyl molecule is such that its migration in the cell is limited and thus reacts quickly with cellular components (Sies, 1985). For the hydroxyl radical to react and oxidize DNA, it must be generated adjacent to the nucleic acid material. Hydrogen peroxide (H₂O₂), a precursor to hydroxyl radical, is less reactive and more readily diffusible and thus more likely to be involved in the formation of oxidized bases (Guyton and Kensler, 1993; Barber and Harris, 1994). Peroxynitrite, another strong cellular oxidant, is formed from the coupling of nitric oxide and superoxide (Beckman et al., 1990; Koppenol et al., 1992). As with H₂O₂, peroxynitrite is diffusible between cells and is taken up by active transport mechanisms into cells (Radi, 1998). Equally important to the induction of mutation by reactive oxygen species is the fact that nitric oxide and superoxide are produced in activated macrophages, and as such, it is likely that peroxynitrite is formed in proximity to these cells. The DNA damaging capability of peroxynitrite may therefore help to explain the reported association between inflammation and mutation (Marnett, 2000).

Oxidation of guanine at the C8 position results in the formation of 8-hydroxydeoxyguanosine (OH₈dG), probably the most studied oxidative DNA adduct. This oxidative DNA lesion results in site-specific mutagenesis, is mutagenic in bacterial and mammalian cells, and produces G→T transversions that are widely found in mutated oncogenes and tumor suppressor genes (Shibutani et al., 1991; Moriya, 1993; Hussain and Harris, 1998). In addition, reactive oxygen species can react with dGTP in the nucleotide pool to form OH₈dG. Therefore, it is postulated
that during DNA replication, OH8dG in the nucleotide pool will be incorporated into DNA opposite dC or dA on the template strand, resulting in A:T to C:G transversions (Cheng et al., 1992; Demple and Harrison, 1994). OH8dG also produces dose-related increases in cellular transformation, which can be prevented by antioxidants, further supporting the role of OH8dG in the carcinogenic process (Zhang et al., 2000). Other oxidative DNA lesions, such as 8-oxo-adenine, thymine glycol, 5-hydroxy-deoxycytidine, as well as several uracil analogs, have been shown to be mutagenic (Wang et al., 1998; Kreutzer and Essigmann, 1998). In summary, oxidized DNA bases appear to be mutagenic and capable of inducing mutations that are commonly observed in neoplasia.

2.6.5. Oxidative stress and cell growth regulation

A role for reactive oxygen species production and oxidative stress has been proposed for both the stimulation of cell proliferation and for cell deletion by apoptosis (Burdon, 1995; Slater et al., 1995). The mechanisms for the involvement of oxidative stress in the induction of the cell proliferation and apoptotic processes are not known, but clearly do not involve a universal mechanism. The effects of reactive oxygen species and oxidative stress within cells appear to be cell specific and dependent upon the form as well as the intercellular concentration of reactive oxygen species. Thus, the involvement of reactive oxygen species in cell growth regulation is complex, and dependent on a number of cellular and biochemical parameters (Klaunig and Kamendulis, 2004).

Reactive oxygen species function to induce cell proliferation during the tumor promotion stage of carcinogenesis (Cerutti, 1985; Bickers & Athar, 2006). Both H2O2 and superoxide anion induce mitogenesis and cell proliferation in several mammalian cell types (D'Souza et al., 1993). Furthermore, a reduction in cellular oxidants via supplementation with antioxidants such as superoxide dismutase, catalase, β-carotene, and flavonoids inhibits cell proliferation in vitro (Alliagana, 1996). Oxidative stress also modulates apoptosis. High concentrations of reactive oxygen species trigger an apoptotic signaling pathway, resulting in cell loss (Dypbukt et al., 1994). A number of endogenous substances (prostaglandins, and lipid hydroperoxides), redox cycling compounds (quinones, adriamycin), and growth factors (transforming growth factor β and tumor necrosis factor α) induce
apoptosis via the generation of reactive oxygen species (Sandstrom et al., 1994; Aoshima et al., 1997). Antioxidants such as N-acetyl cysteine (NAC), glutathione, and dithiothreitol inhibit the apoptotic process, further supporting the link between reactive oxygen species induction and apoptosis (Sandstrom et al., 1994). Although no single mechanism explains the increased cell proliferation and/or inhibition of apoptosis observed following conditions that favor increased cellular oxidants, mounting evidence is emerging that links reactive oxygen species with altered expression of growth regulatory genes.

2.7. Antioxidants

Exposure to free radicals from a variety of sources has led organisms to develop a series of defense mechanisms (Cadenas, 1997). These in vivo defense mechanisms against free radical-induced oxidative stress involve: (i) preventive mechanisms, (ii) repair mechanisms, (iii) physical defenses, and (iv) antioxidant defenses (Valko et al., 2007). Enzymatic antioxidant defenses include superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). They are considered as primary antioxidant enzymes, since they are involved in the direct elimination of active oxygen species. Glutathione-S-transferase (GST), glutathione reductase (GR) and glucose-6-phosphate dehydrogenase (G6PD) are secondary antioxidants which help in the detoxification of reactive oxygen species by decreasing peroxide levels (e.g., GST) or by maintaining a steady supply of metabolic intermediates like glutathione and NADPH for the primary antioxidant enzymes. The non-enzymatic small molecules include sulphydryl compounds such as glutathione (GSH) ascorbic acid, flavonoids and other antioxidants (Sun, 1990). Under normal conditions there is a balance between both the activities and the intracellular levels of these antioxidants. This balance is essential for the survival of organisms and their health.

2.7.1. Biochemistry of antioxidant enzymes

**Superoxide Dismutase (SOD) (E.C 1.15.1.1)**

SOD was first discovered by McCord and Fridovich in 1969 (McCord and Fridovich, 1969). The enzyme is believed to be present in all oxygen metabolizing cells but lacking in most obligate anaerobes, presumably because its physiological function is to provide a defense against the potentially damaging reactivities of the
superoxide radical ($O_2^\cdot$) generated by aerobic metabolic reactions (McCord et al., 1971). This enzyme catalyzes the reaction

$$O_2^\cdot + O_2^\cdot \rightarrow H_2O_2 + O_2$$

Four different forms of SOD have been found to date (Fridovich, 1974): two containing copper and zinc, one manganese and one iron. CuZnSOD is found in the cytosol of most eukaryotic cells (Fridovich, 1975), a different form of CuZnSOD is found in extracellular fluids (Marklund, 1982; Marklund, 1984). MnSOD is located in the mitochondrial matrix as well as in bacteria, while FeSOD is present in many aerobic bacteria (Fridovich, 1974). FeSOD and MnSOD share considerable homology, they are very different from CuZnSODs except in their activity (Banister et al., 1987). Diminished amounts of MnSOD have been found in almost all the tumors examined. Lowered amounts of the CuZnSOD have been found in many, but not all tumors. At the same time tumors have been shown to produce superoxide radicals, therefore, diminished enzyme activities along with radical production may lead to many of the observed properties of cancer cells (Oberley and Buettner, 1979). Numerous studies in cultured cells suggested that MnSOD might function as a new type of tumor suppressor gene (Amstad et al., 1997; Zhao et al., 2001; Zhao et al., 2002).

**Catalase (CAT) (E.C 1.11.1.6)**

Catalase is one of the oldest known enzymes; it was named by Loew in 1901 (Percy, 1984). The enzyme catalyzes the reaction

$$2 H_2O_2 \rightarrow 2H_2O + O_2$$

Most aerobic cells contain this enzyme. In animals, CAT is present in all major body organs, being especially concentrated in liver and erythrocytes. At the subcellular level, CAT is found mostly in peroxisomes (80%) and cytosol (20%) (Sun, 1990). Most purified catalases have been shown to consist of four protein subunits, each of which contains a haem group bound to its active site (Halliwell and Gutteridge, 1990). Catalase activity is found to be low in many animal tumor cell lines (Bozzi et al., 1976). Animal studies have also shown lower CAT activity in tumor than in normal tissue (Tisdale and Mahmoud, 1983).
**Glutathione-S-Transferase (GST) (E.C 2.5.1.18)**

GSTs are a family of enzymes that utilise glutathione in reactions contributing to the transformation of a wide range of compounds, including carcinogens, xenobiotics, therapeutic drugs and products of oxidative stress (Valko et al., 2007). They were originally observed in the catalysis of the first step in the formation of the mercapturic acids (Booth et al., 1961). GSTs occur in substantial quantities in liver and other mammalian tissues e.g., erythrocytes and intestines (Marcus et al., 1978).

A large number of studies have established an association between cancer incidence and various disorders of GSH-related enzyme functions, alterations of GSTs being most frequently reported (Pastore et al., 2003).

**Glutathione Reductase (GR) (E.C 1.6.4.2)**

GR was initially observed in livers from various animals by Hopkins and Elliot in 1931 (Hopkins and Elliot, 1931) and later isolated from ox, sheep and rabbit liver by Mann in 1932 (Mann, 1932). The enzyme catalyses the reaction:

\[
\text{GSSG} + \text{NADPH} + \text{H}^+ \rightarrow 2\text{GSH} + \text{NADP}^+
\]

The enzyme is found in cytosol and mitochondria. GR can also catalyse reduction of certain mixed disulphides, such as that between GSH and coenzyme A. Glutathione activity has been found to be variable in different tumors, however it was found to be lowered in most of them. In a wide variety of mouse tumors, GR activities were, in general, lower in the tumors as compared to corresponding normal tissues (Tisdale and Mahmoud, 1983).

**Glutathione (GSH)**

Glutathione, the most abundant low molecular-weight thiol in mammalian cells, is present in reduced (GSH) and oxidized (GSSG) forms (Meister and Anderson, 1983). The main protective roles of glutathione against oxidative stress are: (i) glutathione is a cofactor of several detoxifying enzymes against oxidative stress, e.g., glutathione peroxidase, glutathions transferase and others; (ii) GSH participates in amino acid transport through the plasma membrane; (iii) GSH scavenges hydroxyl radical and singlet oxygen directly, detoxifying hydrogen peroxide and lipid peroxides by the catalytic action of glutathione peroxidase; (iv) GSH is able to regenerate the most important antioxidants, vitamin C and E, back to their active
forms; glutathione can reduce the tocopherol radical of vitamin E directly, or indirectly, via reduction of semidehydroascorbate to ascorbate (Masella et al., 2005).

The capacity of GSH to regenerate the most important antioxidants is linked with the redox state of the glutathione disulphide-glutathione couple (GSSG/GSH) (Pastore et al., 2003). This ratio is normally closely regulated. Disruption of this ratio is involved in several cellular reactions involved in signal transduction and cell cycle regulation under conditions of oxidative stress (Schafer and Buettner, 2001). It is therefore presumed that decreased GSH would contribute to cell death only when oxidative stress becomes prolonged, and cellular systems are not sufficient to counteract the reactive oxygen species mediated insult (Estrela et al., 2006).

Since free radicals are involved in both initiation and promotion stages of carcinogenesis, one may expect that free radical scavengers should function as inhibitors in the neoplastic processes. Much data has been accumulated on this topic. Antioxidants have been shown to inhibit both initiation and promotion in ssarcinogenesis and counteract cell immortalisation and transformation (Sun, 1990).

2.8. Chemoprevention

The term chemoprevention was first introduced by Sporn, 1976, in contrast to chemotherapy, when he referred to the prevention of development of cancer both by natural forms of vitamin A and its synthetic analogues. This strategy seems to be promising for reducing cancer incidence both in well-defined high-risk groups of people and also in the general population. Chemoprevention is now defined as the use of specific agents to suppress or reverse carcinogenesis (Greenwald and Kelloff, 1996). Primary prevention of cancer is one of the key approaches to the control of cancer. It includes (i) avoiding exposure to known cancer-causing agents, (ii) enhancement of host defence mechanisms, (iii) chemoprevention (Kakizoe, 2003). Cancer chemoprevention may target various processes as proposed by Kellof et al., (1996) e.g., prevention of carcinogen binding to DNA, enhancement of DNA repair, scavenging of oxygen radicals etc.

2.8.1. Antioxidants in Cancer Prevention

In the last few years, there has been growing interest in the role played by oxidative reactions in human diseases. Since oxidative stress is generally perceived as one of
the major causes for the accumulation of mutations in the genome, antioxidants are believed to provide protection against cancer. A number of natural and synthetic antioxidants are known to retard chemical carcinogenesis in experimental animal models, and epidemiological studies suggest that a diet rich in plant products containing natural antioxidants may be deterrent to carcinogenecity (Sardas, 2003). The mechanisms of the anticarcinogenic effects of antioxidants are not fully understood. However, the evidence available so far suggest that the most plausible mechanism for the anti-carcinogenic activity of antioxidants are: (1) scavenging of reactive oxygen species, free radicals and electrophiles; (2) possible attenuation of the formation of activated carcinogenic species by phase I biotransformation enzymes and; (3) enhancement of detoxification of electrophiles by inducing phase II detoxification enzymes such as GST and quinone reductase (Sardas, 2003).

Carcinogens administered to animals are absorbed from the gastrointestinal tract or from the site of administration, and are then distributed throughout the body and metabolised in tissues including the liver and target sites. They are detoxified or activated enzymatically, and activated metabolites or ROS then covalently bind to cellular macromolecules thus causing DNA or cellular damage followed by carcinogenesis (Ito and Hirose, 1989). Antioxidants can modify the carcinogenic process at different stages, including (1) alteration of the metabolic activation of a precarcinogen by (a) inhibition of the activating enzyme or (b) alteration of the metabolic pattern of the carcinogen via selective enzyme induction; (2) prevention of the reaction between the ultimate carcinogen and DNA by (a) direct interaction with the carcinogenic species, (b) increased detoxification by antioxidant-inducible enzymes, or (c) competition between carcinogen and antioxidant in the binding process (Wattenberg, 1978a, b, 1981).

In the process of finding the most efficient agents for better prevention and cure of cancer many natural as well as synthetic antioxidants have been studied. Two such antioxidants; quercetin and melatonin and a medicinal plant extract of podophyllum hexandrum, have been extensively studied for their antioxidant properties against different cancers and were used in this study as well.
Melatonin is the main (indole) hormone of the pineal gland synthesised from tryptophan predominantly during the night (Arendt, 1995). Melatonin is critical for the regulation of circadian and seasonal changes in various aspects of physiology and neuroendocrine function (Arendt, 1995; Pevet et al., 2002). Since 1993, when potent antioxidant properties of melatonin were discovered (Tan et al., 1993), many studies have confirmed the ability of melatonin to protect DNA from free radical damage. It was shown to be a very efficient scavenger of free radicals and was found to be more effective than other well known antioxidants such as glutathione and vitamin E (Musatov et al., 1998; Pieri et al., 1994). Rieter et al (1995) reported that melatonin is a potent quencher of hydroxyl radicals. Melatonin in vitro directly scavenges OH (Tan et al., 1993), H2O2 (Barlow-Walden et al., 1995), singlet oxygen (Qi et al., 2001) and inhibits lipid peroxidation (Pieri et al., 1994). Likewise, melatonin can stimulate a number of antioxidative enzymes including SOD (Antolin et al., 1996), GPx (Pablos et al., 1998), GR (Urata et al., 1999) and catalase (Barlow-Walden et al., 1995). It has been shown that melatonin enhances intracellular glutathione levels by stimulating the rate-limiting enzyme in its synthesis, γ-glutamylcysteine synthase, which inhibits the peroxidative enzymes (Pierrefiche and Laborit, 1995). There is evidence that melatonin stabilizes microsomal membranes; thereby probably helping them resist oxidative damage (Karbownik et al., 2001). It has also been shown to increase the efficiency of the electron transport chain and, as a consequence, to reduce electron leakage and the generation of free radicals (Reiter et al., 2001). It was shown that melatonin reduced the formation of 8-hydroxy-2'-deoxyguanosine more effectively than some classic antioxidants (Qi et al., 2001). Thus melatonin acts as a direct scavenger of free radicals with the ability to detoxify reactive oxygen species and indirectly increasing
the activity of the antioxidative defence systems (Reiter et al., 2001; Qi et al., 2001; Tan et al., 2002). These properties allow melatonin to preserve macromolecules including DNA, protein and lipid from oxidative damage resulted from ionising radiation and chemical carcinogen exposure. Being both lipophilic and hydrophilic, melatonin may play an important role in the antioxidant defence system in all cells and tissues of the body (Reiter et al., 1995).

The role of the pineal gland in tumor development has been under intensive study during the last years (Blask, 1993; Bartsch et al., 2001; Vijayalaxmi et al, 2002; Bartsch and Bartsch, 2006). In cancer patients the morphological signs of pineal function is found to decrease and disturbances in the circadian secretion pattern of melatonin were observed (Vijayalaxmi et al 2002; Bartsch and Bartsch, 2006). The inhibitory effect of melatonin is well established in relation to mammary tumors (Cos et al., 2000; Cos et al., 2001; Sanchez-Barcelo et al., 2003) and colon cancer (Anisimov et al., 1997; Anisimov, 2001). There are few data on the effect of melatonin on tumors of other localizations.

2.8.3. Quercetin

Quercetin (3,3',4',5,7-pentahydroxyflavone) is an important dietary flavonoid present in different vegetables, fruits, seeds, nuts, tea, and red wine (Formica and Regelson, 1995; Beecher et al., 1999; Hollman and Katan, 1999). Quercetin is considered an excellent free-radical scavenging antioxidant owing to the high number of hydroxyl groups and conjugated pi orbitals by which quercetin can donate electrons or hydrogen, and scavenge \( \text{H}_2\text{O}_2 \) and superoxide anion (Heijnen, et al 2001). The reaction of quercetin with superoxide anion leads to the generation of the semiquinone radical and \( \text{H}_2\text{O}_2 \) (Metodiewa et al., 1999). Quercetin also reacts with \( \text{H}_2\text{O}_2 \) in the presence of peroxidases, and thus it decreases \( \text{H}_2\text{O}_2 \) levels and
protects cells against H2O2 damage. The study of Quercetin as potential chemopreventer is assuming increasing importance considering its involvement in the suppression of many tumor-related processes including oxidative stress, apoptosis, proliferation and metastasis. Quercetin has also received greater attention as pro-apoptotic flavonoid with a specific and almost exclusive activity on tumor cell lines rather than normal, non-transformed cells (Lugli et al., 2009). Cancer cells are extremely sensitive to quercetin induced apoptosis. These differences between normal and cancerous cells may result from different signaling pathways (Gupta and Panda, 2002; Kuo et al., 2004; Lugli et al., 2005; Ma et al., 2005; Kim et al., 2008; Chien et al., 2009; Shan et al., 2009; Jung et al., 2010). Quercetin triggers apoptosis via the mitochondrial pathway, through the loss of mitochondrial membrane potential, the subsequent release of cytochrome c from mitochondria to cytosol, and the final activation of caspases, such as caspase-3 and caspase-7 (Lee et al., 2006; Yang et al., 2006; Chien et al., 2009).

2.8.4. Podophyllum hexandrum

Antioxidants of plant origin with free-radical scavenging properties have great importance as therapeutic agents in several diseases caused due to oxidative stress (Ramchoun et al., 2009). Plant extracts and phytoconstituents are found effective as radical scavengers and inhibitors of lipid peroxidation (Yildirim et al., 2001; Dash et al., 2007). Plants also have a long history of use in the treatment of cancer (Hartwell, 1982) and it is significant that over 60% of currently used anti-cancer agents are derived in one way or another from natural sources, including plants, marine organisms and micro-organisms (Newman et al., 2003; Cragg et al., 2005). Two clinically active agents, etoposide (VM 26) and teniposide (VP 16-213), which
are semi-synthetic derivatives of the natural product, epipodophyllotoxin (an isomer of podophyllotoxin), may be considered as being more closely linked to a plant originally used for the treatment of cancer (Lee and Xiao, 2005). The *Podophyllum* species (*Podophyllaceae*), *Podophyllum peltatum* Linnaeus (commonly known as the American mandrake or Mayapple), and *Podophyllum emodii* Wallich from the Indian subcontinent, have a long history of medicinal use, including the treatment of skin cancers and warts. The major active constituent, podophyllotoxin, was first isolated in 1880, but its correct structure was only reported in the 1950s. Application of podophyllum resin 25% solution is efficacious in producing significant short-term resolution of HIV related oral hairy leukoplakia (Cobb, 1990; Gowdey et al., 1995). Podophyllotoxin has been used as a precursor to semi-synthetic anti-cancer drugs like etoposide, teniposide and etopophos. These compounds have been used for the treatment of lung and testicular cancers as well as certain leukemias (Stahelin and Wartburg, 1991; Imbert, 1998). The oxidative stress induced by CCl4 in liver, lung and renal tissues has been reduced by ethanolic extract of *P. hexandrum* by strengthening the antioxidant defense system and by scavenging free radicals in rats. (showkat et al., (a)2010; showkat et al., (b)2010)

### 2.9. Chemotherapy with cisplatin

![Cisplatin structure](image)

Chemotherapy is one of the mainstays of medical intervention for cancer. The interest in platinum based antitumor drugs dates back to the 1960s with the serendipitous discovery by (Rosenberg et al., 1965) showing the inhibition of cell division by platinum complexes. Cisplatin has been used effectively in the chemotherapy of recurrent brain tumours and intracranial germ cell tumours (Spence et al., 1992). The cure rate for testicular cancer is now greater than 90% when tumors are promptly diagnosed (Bosl and Motzer, 1997). The optimal manner of administering the drug is not established, although investigators have used various schedules such as bolus injection (Hayes et al., 1977), prolonged infusion (Loo et al., 1978), and fractional daily usage (Einhorn and Donohue, 1977). Cisplatin is a
neutral, water-soluble, coplanar complex. The drug enters the cell by either passive diffusion or active uptake and is converted to its active form through sequential thermal ligand exchange in an aquation reaction replacing its chloride ions with water or hydroxyl groups (Singh and Turro, 2004). Treatment of tumor cells with cisplatin provokes several responses including membrane peroxidation (Sugiyama et al., 1989), dysfunction of mitochondria (Brady et al., 1990), inhibition of protein synthesis and DNA damage (Kharbanda et al., 1995).

A mechanism by which cisplatin exerts its cytotoxicity is through the generation of ROS (Auersperg et al., 1998). The drug is able to generate reactive oxygen species, such as superoxide anion and hydroxyl radical. Masuda et al. (1994) found cisplatin-induced generation of active oxygen radicals in a cell-free system. Along with its usage several inconvenient side effects are included that need to be dealt with carefully in order to maximize antitumor activity and minimize toxicity. A number of rescue agents such as mesna, WR-2721, diethylthiocarbamate and thiosulfate, selenium and bismuth subnitrate have also been used to control cisplatin toxicity; however, the exact role of these agents is not well understood, and they are yet not routinely being used in Pt chemotherapy (Reedijk, 1999). Intrinsic or acquired resistance, its toxicity towards healthy cells (Zhang and Lippard, 2003), reduction of antioxidant plasma level and generation of free radicals in normal cells (Weijl et al., 1998; Yoshida et al., 2003) limit the organotropic profile of the drug.

2.10. Chemotherapy and antioxidant supplementation

Antineoplastic agents have been shown to produce oxidative stress in patients who receive these drugs during cancer chemotherapy (Sangeetha et al., 1990; Faber et al., 1995; Erhola et al., 1996; Weijl et al., 1998). This is evident by the elevation of lipid peroxidation products; the reduction of total radical-trapping capacity of blood plasma; the reduction in plasma levels of antioxidants and the marked reduction of tissue glutathione levels that occurs during chemotherapy (Conklin, 2004). There are considerable in vitro and animal data showing that vitamin C and other antioxidants can protect cells against radiation and chemotherapy (Sonneveld, 1978; Wierenberg et al., 1999; Greggi et al., 2000). It seems likely that they would therefore reduce treatment-related toxicities and there are promising, although not unequivocal, data that this indeed is the case (Legha, 1982; Mills, 1988;...
Antioxidant supplements are useful in conjunction with chemotherapy because they enhance the efficacy of the chemotherapy, as well as alleviate toxic side effects, allowing patients to tolerate chemotherapy for the full course of treatment and possibly at higher doses (Conklin, 2004). One of the main mechanisms of chemotherapy drugs against cancer cells is the formation of reactive oxygen species (ROS), or free radicals. Drugs with free radical mechanisms include but are not limited to alkylating agents (e.g., melphalan, cyclophosphamide), anthracyclines (e.g., doxorubicin, epirubicin), podophyllin derivatives (e.g., etoposide), platinum coordination complexes (e.g., cisplatin, carboplatin) and camptothecins (e.g., topotecan, irinotecan). Unfortunately, these ROS often are the source of serious side effects, as well. Oxidative stress interferes with cellular processes that are necessary for antineoplastic agents to exert their optimal cytotoxicity on cancer cells, and modest levels of oxidative stress have been shown to reduce the cytotoxicity of anticancer drugs. (Lee and Shacter, 1999; Shacter et al., 2000) Thus, the formation of ROS that occurs when anticancer drugs are administered may diminish the effectiveness of the treatment. In addition, since some side effects caused by antineoplastic agents appear to be prevented by certain antioxidants, administering these supplements during chemotherapy may diminish the development of side effects as well as improve the response to therapy. This contention is supported by many preclinical and some clinical studies. (Lamson and Brignall, 1999; Conklin, 2000)

Cisplatin [cis-diamminechloroplatinum (II)] (CDDP) is a potent antineoplastic agent used for the treatment of a wide range of cancers (Saad et al., 2004; Wang et al., 2004). Oxidative stress is one of the most important mechanisms involved in CDDP-induced toxicity. The biochemical basis of cisplatin toxicity is believed to relate to free radicals generated in these tissues (Ravi et al., 1995). Indeed, several antioxidants have been tested, with limited success, as potential agents to ameliorate the destructive actions of this chemotherapeutic drug (Ravi et al., 1995).

The study of Qu as potential chemopreventers, or for chemotherapy, is assuming increasing importance, considering its high toxicity for cancer cells, along with its relatively scarce effects on normal, non-transformed cells (Lugli et al., 2009).
Melatonin, a hormone of the pineal gland has been known to be a chemopreventive, anticancer agent in in vitro studies and experimental animal models (Cos et al., 2005). In pharmacological amounts, melatonin effectively reduces oxidative stress through several mechanisms (Martinez et al. 2005). Considering the low toxicity of melatonin and its ability to reduce the side effects and increase the efficacy of drugs, its use as a combination therapy with anticancer agents seems important and worthy of pursuit.

Natural polyphenolics are reported to contain several biologically active flavonoids, polyphenols and podophyllotoxin glycoside, and the like that might contribute toward enhancing DNA repair (Gao et al., 2006). The therapeutic efficacy of podophyllum hexandrum is predominantly attributed to their properties of free radical scavenging, reduced lipid peroxidation (Gupta et al., 2007), transient metal-chelation (Kumar and goel, 2000) and protection to endogenous defense enzymes (Gupta et al., 2007) and to cellular DNA.

2.11. Stress and chemoprevention

Epidemiological (Fox, 1981; Locke, 1982; Temoshok and Fox. 1984) and experimental (Sklar and Anisman, 1980; Sklar and Anisman, 1981) evidence suggest that stress and ability to cope with stress may play a role in disease processes as well as cure. Psychosocial stressors have been associated with reduced efficacy of cancer therapies (Lichtman et al., 1987; Waxler-Morrison et al., 1991). Other studies have shown that reducing the impact of life-stressors through psychosocial intervention or social support may extend survival time and decrease the toxic side effects of chemotherapy (Burish et al., 1987; Grossarth and Eysenck, 1989). In support of these data, recent animal studies have shown that exposure to rotational stress decreases the anti tumor effects of chemotherapeutic drugs in terms of tumor burden, extent of metastasis and survival time (Pressin et al., 1991; Giraldi et al., 1992; Giraldi et al., 1994). Several factors appear to play a role in mediating stressor-induced alterations in the effectiveness of chemotherapy treatments, including tumor size and growth rates, hormone levels, and/or immune function, or an interaction among these factors (Corbett et al., 1978; Armitage, 1992; Bassukas and Schultze, 1993; Boddy and Idle, 1993; Maclean and Longenecker, 1994). So far, many stressors have been evaluated for their possible effect on chemoprevention and
chemotherapy. (Perissin et al., 1991) investigated the influence of stress on the effects of the antitumor cytotoxic drug cyclophosphamide and the selective non-cytotoxic antimetastatic agent razoxane. In mice bearing Lewis lung carcinoma the application of rotational stress significantly decreased the magnitude of the effects of both drugs. Further studies showed that the curative action of cyclophosphamide was abolished when animals were subjected to restraint stress. The application of restraint stress, which by itself has no effects on survival, completely abolished the increase in survival time caused by the treatment with cyclophosphamide (Zorzet et al., 1998). Within a conceptualized framework of stress and disease, bio-behavioral factors are understood to influence multiple aspects of tumor growth including apoptosis, angiogenesis, invasion, and immunological escape to the metastatic cascade (Antoni et al., 2006). As one example, transferring laboratory mice from group housing to social isolation accelerates growth of induced tumors and attenuates the effects of chemotherapy (Grimm et al., 1996, Kerr, 1997). Although many studies have been carried out to determine the impact of stress on chemotherapy, less data is available to-date on the relevance of psychological factors in determining the outcome of cancer chemoprevention.