Review of Literature
2.1. CARCINOGENESIS

Carcinogenesis is the process by which normal cells are transformed into cancer cells. In today's developed world the problem of cancer is increasing and is now second only to cardiovascular diseases as a cause of death, affecting millions of people worldwide (Jemal et al., 2002). The term cancer refers to more than hundred types of the disease. Almost every tissue in the body can spawn malignancies and some can yield several types (Waris and Ahsan, 2006). The induction of cancer (carcinogenesis) is a multistage process and its stages have been defined experimentally as initiation, promotion and progression (Pitot et al., 1981).

Initiation involves the formation of a mutated, preneoplastic cell from a genotoxic event due to mutation of DNA resulting in the activation of oncogenes and the inactivation of tumor suppressor genes. Initiation is thought to be irreversible, dose-dependent process and consist of a single gene mutation that is caused in most cases by environmental genotoxic agents such as chemicals, radiation and viruses (Bishop. 1991; Marshall, 1991).

Promotion follows initiation and involves the process of gene activation such that the latent phenotype of the initiated cell becomes expressed through cellular selection and clonal expansion. This can occur through a variety of mechanisms including toxicity, terminal differentiation or mitoinhibition of the non-initiated cells and mitogenesis or decrease in apoptosis of the initiated cell population (Slaga 1984; DiGiovanni 1992). While promotion occurs over a long period of time, events of this process are dose-dependent and reversible upon removal of the tumor promotion stimulus (Schulte-Hermann et al., 1994).

Progression, the third stage, involves genetic damage that results in the conversion of benign tumors into malignant neoplasms capable of invading adjacent tissues and metastasizing to distant sites (Slaga, 1984; DiGiovanni 1992). This stage is irreversible which involves genetic instability, changes in nuclear ploidy, and disruption of chromosome integrity (Klaunig & Kamendulis, 2004).

Carcinogenesis depends on inherited and acquired susceptibility factors, on exposure to initiation factors i.e., exogenous and endogenous carcinogens and on promotion and progression factors (Walaszek et al., 2005). It is generally accepted that the causes of cancer could be physical (e.g., radiation), chemical (carcinogens), and viral (e.g.,...
Review Of Literature

oncogenes). The combined effect of stimulatory factors (e.g., hormones, cytokines), stress mediators (oxygen radicals) and exogenous aggressions (viruses, radiation and chemical carcinogens) can affect the control of cellular proliferation and lead to tissue transformation (Garry et al., 2000). The mechanism by which certain carcinogens and radiation cause carcinogenesis is believed to be mediated by free radicals. Numerous antecedent studies have demonstrated that ROS participate in all the three stages of carcinogenesis (Li et al., 1997). Much of the evidence has come from the fact that antioxidants that scavenge free radicals directly, or that interfere with the generation of free radical-mediated events, inhibit the neoplastic process (Sun, 1990). Extensive evidence is documented which indicates that reactive oxygen species are pervasive mutagens, e.g., oxy radicals (Hasan et al., 1984), hydrogen peroxide (Kenese et al., 1989), as well as various endogenous ROS (Simic et al., 1989). ROS can also interfere with cell signaling by altering protein kinase cascades and transcription factors, ultimately leading to tumor development (Kovacic & Jacintheo, 2001). There is convincing evidence that cellular oxidation states, i.e., the relative levels of ROS, antioxidant defense entities, and radical scavengers can promote initiated cells to neoplastic growth (Cerutti, 1985; Kenseler & Tarffe, 1986). Investigations dealing with involvement of ROS have continued into recent years (Abdi & Ali, 1999; Huang et al., 1999). One of the most powerful tumor promoters is phorbol myristate acetate and it is well established that its activation is accompanied by appearance of ROS, including superoxide (Delclos & Blumberg, 1982), as well as hydroxyl radicals and lipid peroxidation (Nakamura, 1985).

2.2. STRESS AND CANCER

The term ‘stress’ although having a very broad meaning, generally describes a state of disturbed homeostasis, harmony and equilibrium (Levine & Ursin, 1991; Weiner, 1992; Johnson et al., 1992). The disturbing forces or inputs, which result into such state are known as stressors, while the counteracting forces as adaptation. 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.
Stress is a common phenomenon which is attracting increasing attention as it is found to affect many aspects of physiology and has an important influence on health and diseases. Stress has long been associated with gastric and duodenal ulcers, hypertension, cardiovascular and cerebrovascular diseases, cancer and aging (Cooper 1984; Chrousos et al., 1995; Csermely, 1998). Long-term exposure to stress has detrimental effects on several cell functions in many species, including humans (Kovacs e 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 & 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 & Gosse, 1973; Benchfield et al., 1978; Goldman & Vogel, 1984; Laconi et al., 2000). However, results are contradictory in that both exacerbation and attenuation of tumor development. Animal experiments have generally documented a positive association between the two variables (Sklar & Anisman, 1979; Sklar & Anisman, 1980; Steplewski et al., 1985; Matsukawa et al., 1997), although some studies have led to opposite conclusions (Amkraut & Solomon, 1972; Pradhan & Prabhati, 1974; Justice, 1985). For example, foot shock enhanced the growth of transplanted neoplastic cells in mice (Sklar & Anisman, 1979) and a similar pattern of results have been reported when psychosocial stress (isolation) was used in animals bearing grafted tumors (Dechambre & 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 & 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 & Ray, 1974; Bhattacharya & Pradhan, 1979; Ray & Pradhan, 1979). Animals exposed to electroconvulsive shock and cold showed better rejection and reduced growth of murine sarcoma and lymphoma tumors (Amkraut & 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 & 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 & 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). 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 & Mori, 1999; Matsumoto et al., 1999; Olivenza et al., 2000; Zaidi et al., 2005). 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

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 & 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 & 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 & Machida, 2002). Antioxidant defense status in peripheral tissues is thus influenced by immobilization stress and tissue specific regulation mechanisms of antioxidants exist in rats.

2.2.2. Stress and DNA Damage and Repair

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). 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. 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). Glaser and coworkers (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 & 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 & 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 & 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 & 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 & 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
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Review Of Literature

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 & Augusto, 1999). More than forty years ago, hydrogen peroxide was reported to be an initiator (Kovacic, 1959). In fact, many carcinogens indirectly generate H$_2$O$_2$, which in turn brings about increased amounts of ROS (Kovacic, 1959). More recent experimentation has elucidated many specifics concerning H$_2$O$_2$-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 (-CH$_3$) (Hix & 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: alkoxyl, peroxy, and hydroxyl. These ROS have been implicated in DNA cleavage (Adam et al., 1998) as well as oxidation of DNA bases (Simandan et al., 1998).

2.3.3. Haloalkanes (HAs)
Carbon tetrachloride (CCl$_4$) and other haloalkanes (HAs) are widely used industrial solvents. The carcinogenicity and toxicity associated with CCl$_4$ exposure is well established (Frezza et al., 1994; Brennan & Schiestl, 1998). Many HAs, including CCl$_4$, are procarcinogens that become activated via a sequence of redox reactions whereby
Review Of Literature

CYP450 acts as the principal catalyst in the dehalogenation (Yao et al., 1994). In a study involving $^{18}$O, 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).

$$\text{CCL}_4 \xrightarrow{e^-} \text{CCL}_4^- \xrightarrow{\text{Cl}} \text{Cl}_3 \text{C}^- \xrightarrow{\text{O}_2} \text{Cl}_3 \text{COO}^- \xrightarrow{\text{H}^+} \text{Cl}_3 \text{COOH} \rightarrow \text{Oxy Radicals}$$

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 & 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 & 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
Review Of Literature

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 (Schut & Synderwine, 1999; Adamson et al., 1996). 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 & 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 & 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., 1987).
Biological systems have developed methods for dealing with electrophilic xenobiotics produced during Phase-I metabolisms; they are generally classified as Phase-II enzymes (Fig.3). 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).

**Overview of Metabolic Activation of Xenobiotics**

![Diagram](image)

*Fig.3. Phase I metabolism can either activate or detoxify. Phase II metabolism makes the metabolite more polar so it can be excreted, and usually detoxifies (with some exceptions).*
2.4. DMBA (7, 12-dimethylbenz (a) anthracene)

DMBA, a member of the polycyclic aromatic hydrocarbons, is present in the environment as a product of incomplete combustion of complex hydrocarbons. It is also present in some amount in cigarette smoke (Rodgman et al., 2000; Bhuvaneswari et al., 2004). DMBA has been extensively used as a prototype agent in mutation research and cancer research. The main target sites for the potent carcinogenicity of this agent in rodents are the skin and the mammary gland (Huggins et al., 1961; Gruenstein et al., 1966). Being an indirect carcinogen, DMBA requires metabolic activation to become a carcinogen. DMBA is metabolized by CYP4501A1 in liver microsomes and by CYP4501B1 in primary bone marrow to form diol epoxides and toxic ROS (Guerin, 1978). 7,12-DMBA is converted to the proximate carcinogenic metabolite, namely 7,12-DMBA-3, 4-oxide by CYPs (Fig. 4). This epoxide is hydrolysed by epoxide hydrolase to form 7,12-DMBA-3, 4-diol, which is finally oxidized again by CYPs to the ultimate carcinogenic metabolite 7,12-DMBA-3, 4-diol-1, 2-epoxide (Conney, 1982; Luch et al., 1999). Indeed, a general correlation exists between the strength of a series of dihydrodiol epoxides as initiators, and their chemical potential to form the benzylic carbonium ion, which is a reactive, electrophilic intermediate that binds to nucleophiles (Jerina et al., 1976). DMBA covalently modifies DNA by reaction of its bay region diol epoxide with exocyclic amino groups of deoxyguanosine and deoxyadenosine.

![Fig. 4. Metabolic activation of 7,12-DMBA to bay region epoxide by P450 and epoxide hydrolase](image)

2.4.1. DMBA induced carcinogenesis

DNA adduct formation is generally accepted as a critical step in the mechanism by which polyaromatic hydrocarbons, including 7,12-DMBA, cause mutations resulting in
induction of cancer in the target organs (Dipple et al., 1984). Many studies have shown that these carcinogenic hydrocarbons after being metabolized by CYP450 (Kadlubar & Hammons, 1987; Christou et al., 1987), as an essential first step, are transformed to DNA binding meso-region benzylic electrophilic metabolites that result in carcinogenesis (Flesher & Sydnor, 1971; Flesher & Sydnor, 1973; Surh et al., 1989; Flesher et al., 1997a,b). Cavelieri and group, however, proposed that one electron oxidation is the predominant mechanism of activation for the most potent PAH including DMBA, benzo[a] pyrene and 3-methyl cholanthrene.(Ramakrishna et al., 1992). Mammalian peroxidases, including prostaglandin H synthase (Degen et al., 1982; Josephy et al., 1983; Boyd & Eling, 1984; Cavalieri et al., 1988), and cytochrome P-450 (Hanzlik & Tullman, 1982; McDonald et al., 1982; Augusto et al., 1982; Burka et al., 1985; Cavalieri et al., 1988) catalyse one-electron oxidation, and this mechanism is also involved in the binding of PAH to DNA (Cavalieri et al., 1983; Devanesan et al., 1987; Rogan et al., 1988; Cavalieri et al., 1990). Evidence for activation of DMBA by one-electron oxidation comes from observations concerning benz[a]anthracene as well as the chemical properties of the DMBA radical cation (Dipple, 1976). The two major DMBA-DNA adducts formed by this mechanism are depurination adducts in which specifically the 12-CH$_3$ group is bound to the N-7 of adenine [7-MBA-12-CH$_2$ – N$_7$ Ade] or guanine[7-MBA-12-CH$_2$ - N$_7$ Gua] (Ramakrishna et al., 1991). These two adducts constitute almost 99% of the total adducts (Ramakrishna et al., 1992). Activation of DMBA by one-electron oxidation and its specific reaction at the 12 CH$_3$ group with DNA nucleophiles is in agreement with the results of several relevant carcinogenicity experiments.

The interaction product between the chemical and DNA, known as a DNA adduct, is the precursor lesion for mutation. Alteration in DNA sequence through genotoxin exposure can give rise to myriads of alterations within the cell including switching on or off of genes, aberrant protein expression or alterations in cell cycle control (Garner, 1998). Recent evidence supports the concept of mutated genes, which once mutated give rise to genetic instability. For example, if a mutation occurring in a gene specifying a DNA-repair enzyme will result in lack of DNA repair fidelity ensuing cascade of new mutations which will lead to further cell dysregulation and dysfunction. Thus, from a single genetic event, a much larger group of mutations can result (Bohr, 1995).
Chromosome breaks were also seen in bone marrow cells of Chinese hamsters after subcutaneous or intraperitoneal inoculation of DMBA (Kato et al., 1969). There are a number of possible mechanisms by which DMBA could produce chromosome breakage; one is binding of the bulky diol epoxides to DNA and the second involves the induction of a pre-oxidant state in treated cells (Cerutti, 1985). ROS formed during DMBA metabolism can diffuse from the site of generation to other targets within the cells or even propagate the injury outside to intact cells. These ROS produce deleterious effects by initiating lipid peroxidation directly or by acting as second messengers for the primary free radicals that initiate lipid peroxidation (Das, 2002). Evidence for a role of reactive oxygen species in chromosome breakage was found in human leukocyte cultures treated with DMBA (Shamberger et al., 1973). DMBA induced about a 3-fold increase in chromosome breaks and these could be reduced by as much as 64% by the presence of antioxidants during DMBA treatment. The antioxidant effect is corroborated by the observation that peroxidation of unsaturated lipids increases in mouse skin ∼3-fold by 20 days after a single application of DMBA (Shamberger, 1972). Further studies showed that antioxidants inhibit experimental carcinogenesis induced by DMBA, thus confirming the involvement of ROS (Sharma et al., 2004; Sultana & Saleem, 2004). DMBA, a complete skin carcinogen, induces substantial oxidative effects that are similar to those of TPA. A prominent effect is oxidative modification of DNA bases, which occurs during the same time period that base-DMBA adducts are formed. Major events associated with tumor promotion are of longer duration after multiple DMBA treatments than those induced by TPA (Frenkel et al., 1995). Moreover, it causes formation of the same types of oxidized DNA bases as does ionizing radiation (Dizdaroglu, 1985; Frenkel, 1992) an archetypical free radical generating complete carcinogen. Several applications of DMBA provide an added complexity to interactions between concomitantly formed carcinogen-DNA base adducts (initiation) and oxidized bases (promotion). These concurrent interactions of initiating and promoting processes then may be the key to complete chemical carcinogenesis.

2.5. REACTIVE OXYGEN SPECIES (ROS):
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 & 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 (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 (¹O₂), hydrogen peroxide (H₂O₂), and the highly reactive hydroxyl radical (·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 byproducts 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), sulphydryl groups in proteins (Knight, 1995) and cross-linking/fragmentation of ribonucleoproteins (Waris & Alam, 1998) (Fig.1). 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 & 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 & 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).

**Fig.1.** Pathways illustrating the sources of reactive oxygen species and its role in the development of cancer.
2.5.1. 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 & 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.

\[
\text{Substrate-H + O}_2 + \text{RH}_2 \rightarrow \text{Substrate-OH} + \text{R} + \text{H}_2\text{O} \quad (I)
\]

*Mono-oxygenation of substrate by cytochrome P450*

Molecular oxygen often undergoes a single electron reduction to form the superoxide radical anion (SO⁻) (Hauptmann & Cadenas, 1997). The production of superoxide occurs mostly within the mitochondria of a cell (Cadenas & 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⁻) alkoxyl (RO). Such entities, including SO⁻ itself, can cause several internal anomalies, including: enzyme
inactivation, lipid and protein peroxidation, and DNA oxidation (Halliwell & 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.

$$2O_2^- + 2H^+ \rightarrow 2H_2O_2 + O_2$$ (II)  

*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 & Fridovich, 1994). The released Fe²⁺ can participate in the Fenton reaction, generating highly reactive hydroxyl radical. Thus under stress conditions, O₂⁻ acts as an oxidant of [4Fe-4S] cluster-containing enzymes and facilitates OH production from H₂O₂ by making Fe²⁺ 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.

$$Fe(II) + H_2O_2 \rightarrow Fe(III) + HO^- + HO^-$$ (III)  

*Fenton reaction*

$$Fe(III) + O_2^- \rightarrow Fe(II) + O_2$$

$$Fe(II) + H_2O_2 \rightarrow Fe(III) + HO^- + HO^-$$

$$O_2^- + H_2O_2 \rightarrow HO^- + OH^- + O_2$$ (IV)  

*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 in vivo half-life of
Review Of Literature

approx. 10^-9 s (Pastor, 2000). Thus when produced 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 & 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 & Dix, 1991). The LOOH-dependent pathway of HO2• initiated fatty acid peroxidation may be relevant to mechanisms of lipid peroxidation initiation in vivo (Valko et al., 2007).

2.5.2. 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.

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

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.

$$\text{Lipid} + O_2 \rightarrow \text{Lipid-O}_2^\cdot$$

(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 $\text{R-OOH}$ (Halliwell & Gutteridge, 1984)

$$\text{Lipid-O}_2^\cdot + \text{Lipid-H} \rightarrow \text{Lipid-O}_2 \cdot \text{H} + \text{Lipid}$$

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; Kaschnitz & Hatefi, 1975; Gutteridge, 1977; Aust & 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-0$_2$H), in a similar way to its reaction with $H_2O_2$ to give alkoxy (lipid-O') radicals.

$$\text{Lipid-O}_2 \cdot + \text{Fe}^{2+}\text{-complex} \rightarrow \text{Fe}^{3+}\text{-complex} + \text{OH}^\cdot + \text{lipid-O}'$$

(Fenton reaction)

With an iron (III) compound a peroxy (lipid- O$_2$) radical will form:

$$\text{Lipid-O}_2 \cdot + \text{Fe}^{3+}\text{-complex} \rightarrow \text{lipid- O}_2 + \text{H}^+ + \text{Fe}^{2+}\text{-complex}$$

Both alkoxy and peroxy radicals stimulate the chain reaction of lipid peroxidation by abstracting further hydrogen atoms.
2.5.3. 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 & 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 (Golding et al., 1989). The dimer of MDA is approximately equipotent to monomeric MDA as a mutagen (Riggins & Marnett, 2001). MDA reacts with nucleic acid bases at physiological pH to form adducts to dG, dA and dC (Fig.2) (Seto et al., 1981; Nair et al., 1984; Marnett et al., 1986; Stone et al., 1990 a,b).

![Fig 2. Generation of monomeric adducts from MDA reaction with DNA.](image)

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 \(-\text{NH}_2\) or \(-\text{SH}\) 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 \(-\text{SH}\) 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 (Halliwell & Gutteridge, 1999). 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.

2.5.4. 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 & 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 \(G \rightarrow T\) transversions (Higinbotham et al., 1992; Du 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
Review Of Literature

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 & 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 & Kensler, 1993; Barber & 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...
Review Of Literature

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 & 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 & 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 & Essigmann, 1998). In summary, oxidized DNA bases appear to be mutagenic and capable of inducing mutations that are commonly observed in neoplasia.

2.5.5. 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 & 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 \textit{in vitro} (Alliagnana, 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.

2.6. ANTIOXIDANTS

Exposure to free radicals from a variety of sources has led organisms to develop a series of defense mechanisms (Cadenas, 1997). These \textit{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 sulphhydryl 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
Review Of Literature

levels of these antioxidants. This balance is essential for the survival of organisms and their health.

2.6.1. Biochemistry of antioxidant enzymes

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

SOD was first discovered by McCord and Fridovich in 1969 (McCord & 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$_2^-$) generated by aerobic metabolic reactions (McCord et al., 1971). This enzyme catalyzes the reaction

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

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 & 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

\[ 2 \text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2 \]
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 hem group bound to its active site (Halliwell & 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 & 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 & Elliot, 1931) and later isolated from ox, sheep and rabbit liver by Mann in 1932 (Mann, 1932). The enzyme catalyses the reaction:

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

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 & 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 & 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/2GSH) (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 & 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 carcinogenesis and counteract cell immortalisation and transformation (Sun, 1990).

2.7. 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 & 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.

2.7.1. 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 carcinogeneity (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 & 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
detoxication 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; melatonin and resveratrol, have been extensively studied for their antioxidant properties against different cancers and were used in this study as well.

2.7.2. Melatonin (N-acetyl-5-methoxy-tryptamine)

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 (Pierl 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 & 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.
Review Of Literature

(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 & 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 & 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.

2.7.3. Resveratrol (3,4', 5-trihydroxy-trans-stilbene)

The plant polyphenol resveratrol has been classified as a phytoalexin, because it is synthesised in spermatophytes in response to certain types of stress, including injury, UV irradiation or fungal attack (Langcake & Pryce, 1977; Hain et al., 1990). Resveratrol naturally occurs in grapes (Langcake & Pryce, 1976; Romero-Perez et al., 1999; Roldan et al., 2003), wine (Siemann & Creasy, 1992), and peanuts (Sobolev & Cole, 1999; Ibern-Gomez et al., 2000; Sanders et al., 2000). It has been shown to inhibit platelet aggregation and eicosanoid synthesis (Pace-Asciak, 1995), to interfere with arachidonate metabolism (Kimura, 1985), to exert strong inhibitory effect on reactive oxygen species produced by human polymorphonuclear leukocytes (Rotondo, 1998), and to be antioxidant more powerful than vitamin E in preventing low-density lipoprotein (LDL) oxidation (Frankel, 1993). Lately, due to its ability to serve as an effective antioxidative agent (Fremont, 2000), resveratrol has received wide attention. It inhibits cellular events associated with tumor initiation, promotion/progression (Cadenas & Barja. 1999). Recently, resveratrol was found to possess cancer prevention activity in several animal cancer models, such as by blocking the development of preneoplastic lesions in carcinogen-treated mouse mammary glands (Jang et al., 1997).
Chemopreventive strategies include the inhibition of phase I enzymes responsible for activating xenobiotics and the induction of phase II enzymes that conjugate these activated compounds to endogenous ligands (e.g., glutathione). Resveratrol, being an exogenous lipophilic compound, can cross plasma membrane, be subjected to cellular metabolism and it possibly interacts with phase I enzymes (Signorelli & Ghidoni, 2005). Resveratrol inhibited human recombinant CYP 450 in vitro (Yu et al., 2003). Moreover, it inhibited CYP450 activity from mouse or human liver microsomes (Ciolo & Yeh, 1999; Mikstacka et al., 2002). Jang et al. (1997) found that resveratrol reduced the insurgence of preneoplastic lesions in mouse mammary gland cultures and decreased the incidence of tumor formation in mice treated with 7,12-dimethylbenz(a)anthracene (DMBA) used as tumor initiator, in combination with phorbol esters used as tumor promoter. Since DMBA requires bioactivation by phase I enzymes (Shou et al., 1996), the antitumoral activity of resveratrol in vivo includes prevention of the initiation phase of carcinogenesis by inhibiting phase I enzymes. Resveratrol was further shown to induce phase II enzymes such as UDP-glucuronyltransferase and NAD(P)H:quinone oxidoreductase in mouse epidermis (Szaefer et al., 2004). These data strengthen the hypothesis that resveratrol may be used in cancer prevention.

2.8. STRESS AND CHEMOPREVENTION

Epidemiological (Fox, 1981; Locke, 1982; Temoshok & Fox, 1984) and experimental (Sklar & Anisman, 1980; Sklar & 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 & 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; 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 & Schultze, 1993; Boddy & Idle, 1993; Maclean & Longenecker, 1994). So far, many stressors have been evaluated for their possible effect on chemoprevention and chemotherapy. Perissin and coworkers investigated the influence of stress on the effects of the antitumor cytotoxic drug cyclophosphamide and the selective non-cytotoxic antimetastatic agent razoxane (Pressin et al., 1991). 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). 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.