Discussion
DISCUSSION

The recent world cancer report released by WHO observes that, world cancer rates are set to double by 2020 (Eaton, 2003). Cancer is emerging as a major problem globally; both in more developed and in less developed countries. Furthermore, cancer mortality in the world as a whole, is more than twice, that in developing countries, a factor the report attributes to the earlier onset of the tobacco epidemic, earlier exposure to occupational carcinogens and the western diet and life style. Carcinogenesis is a multistage disease process that has been classified into initiation, promotion and progression stages; and each stage probably involves both genetic and epigenetic changes (Bishop, 1991). Metabolic activation of carcinogen is a free-radical-dependent reaction. DNA damage mediated by free radicals plays a critical role in carcinogenesis (Guyton and Kensler, 1993; Feig et al., 1994). In biological systems, damaged DNA is repaired enzymatically and cells regain their normal functions. However, misrepair of DNA damage may result in mutations such as base substitution and deletion, leading to carcinogenesis (Poulsen et al., 1998).

Many currently used anti-cancer drugs are known to be mutagenic and substantially increase the risk to the patient, of secondary tumors, of reduced fertility or of birth defects in children born subsequent to the therapy (e.g., Sorsa et al., 1985). A considerable amount of data are available, analyzing
point mutations, chromosomal aberrations or DNA-repair activities of these drugs, and many of the mutagenic effects may be due to such events. However, other genetic activities may also be important, although there is generally less information available on these.

Several clinically used anti-cancer drugs such as Hydroxyurea (Zimmermann, 1971), Anthramycin (Hannan et al., 1978), Bleomycin (Hannan and Nasim, 1978a), Daunomycin (Hannan and Nasim, 1978b), Cisplatinum (Hannan et al., 1984), Cyclophosphamid (Arni and Muller, 1983) and Mitomycin C (Holliday, 1964) have caused mitotic crossing-over in various assays.

It was the aim of the present study to evaluate the genotoxic and biochemical effects of the anti-cancer agents, Cisplatin, Etoposide, Flutamide and Vincristine sulphate by using standard and time-tested procedures on: mice germ cells, by the observation of the level of abnormal sperm induced by chemical treatment; on somatic cells, by studying the chromosomal aberrations, as well as on peripheral blood erythrocytes for induction of micronuclei. Biochemical tests on the changes in the enzyme levels due to the presence of the drugs were also performed.

The mouse sperm morphology assay (Wyrobek and Bruce, 1975) has been proposed as an in vivo methodology to study the genotoxic effects of
chemical or physical agents on germ cells due to the fact that: (1) the morphology of the sperm is genetically determined (Wyrobek and Bruce, 1978); (2) most mutagens are able to induce sperm abnormalities in mice (Wyrobek and Bruce, 1975; Bruce and Heddle, 1979; Topham, 1979); (3) teratospermia are transmissible to the F1 offspring of male mice exposed to chemical or physical treatment (Wyrobek and Bruce, 1978; Topham, 1980a). In fact, the analysis of sperm morphology provides a useful tool for the detection of testicular toxins (Topham, 1980b).

Chemical mutagens induce a variety of sperm abnormalities. These may be detected at the chromosome level, for example, in Y chromosome non-disjunction (Kapp, 1978), or at the cellular level, in which morphological aberrations of sperm-heads (Wyrobek and Bruce, 1975) or aspermia may be observed (Cacheiro and Russel, 1980). The genetic transmission of some of these effects has been reported (Wyrobek and Bruce, 1978).

The bone marrow micronucleus (MN) assay has been used extensively to detect acute chromosomal damage occurring in erythroblasts (Schmid, 1976; Heddle, 1973). It has been widely used as a screening test, for detecting clastogenic and spindle-damaging effects of chemicals in vivo (Schmid, 1975; Heddle et al., 1983; MacGregor et al., 1987; Mavournin et al., 1990; Miller et al., 1991; Gudi et al., 1992). However, this test does not allow us to monitor the same treated animals continuously, because the animals have
to be killed to obtain the bone marrow samples. Until recently, blood samples were thought to be unsuitable for this purpose because the spleen was believed to remove micronucleated erythrocytes from the peripheral circulation of many species (Schalm, 1970), including the mouse (Von Ledebur and Schmid, 1973). The finding that micronucleated erythrocytes persist in the circulation of the mouse (Schlegel and MacGregor, 1982) now permits the detection of both, acute (MacGregor et al., 1980) and cumulative (Schlegel and MacGregor, 1983) chromosomal damage, through the examination of peripheral blood smears.

Advantages of sampling from peripheral blood rather than bone marrow are: (1) sample preparation is simple and rapid; (2) animals in ongoing studies need not be sacrificed to be sampled; (3) multiple samples can be taken from the same animals; (4) retrospective analysis can be performed on blood smears taken during toxicity studies; and (5) cumulative cytogenetic damage can be assessed since micronucleated erythrocytes persist in the circulation.

In mice, unlike rats and humans, the MNEs have a normal life span in blood circulation and are not captured and destroyed by spleen (Schlegel and MacGregor, 1984).

Micronuclei in bone marrow or peripheral blood erythrocytes provide a
useful index of chromosomal breakage or anaphase lag in erythroblasts (Heddle et al., 1983; MacGregor et al., 1980; Schmid, 1976; Schlegel and MacGregor, 1982, 1983). During the last decade it has been shown that the analysis of MN in polychromatic erythrocytes (PCE) of mouse peripheral blood has the same sensitivity compared with the bone marrow MN test. The main difference is that the maximum of the MN frequency in peripheral blood usually occurs 24 hours later than in bone marrow. Young erythrocytes in mouse peripheral blood are used increasingly as alternative target cells for detecting clastogenic or spindle damaging effects of chemicals (Hayashi et al., 1990; MacGregor et al., 1980, 1990; Cao et al., 1992).

One of the advantages of the MN test as an in vivo cytogenetic assay, compared with conventional metaphase chromosomal aberration analysis, is the simple and rapid scoring. Qualitatively, the evaluation of MN induction does not have to rely on MNPCE data, but can be based on observations of total erythrocytes as well (Yamamoto and Kikuchi, 1980).

In surveys of mutagenic properties of anti-cancer drugs such as that by Sorsa et al., (1985), it is commonly implied that alkylating agents are the most potent mutagens and carcinogens. Although some of these agents may not themselves be very effective point mutagens (Anonymous, 1981), there is a high probability that they will be combined with a point mutagen in their clinical scheduling (Anonymous, 1981; Sorsa et al., 1985). Treatment with a
point mutagen is most likely to cause recessive mutations that will be masked by the corresponding wild-type allele in diploid cells. If such treatment is followed by treatment with an agent preferentially causing mitotic crossing-over, then such heterozygous genes could be brought into the homozygous state, potentially leading to the expression of various types of mutation.

The procedures that are currently available for the detection of transmissible genetic damage in mammals (e.g., tests for heritable translocations or for specific-locus mutations) require large numbers of animals and/or are costly in time and effort.

Although there is a great deal of published literature on mutagenesis by anti-cancer drugs (e.g., Anonymous, 1981), it is often difficult to get relative values, which might rationally be used in the selection of drugs, in order to minimize genotoxic activity. In practice, at present, there is not usually sufficient choice of agents appropriate to various tumor types that might permit a secondary drug selection on the basis of mutagenic or recombinogenic properties. However, with an increasing number of drugs coming onto the market, the situation may change.

In addition to environmental mutagens, our DNA is subject to assault by damaging agents formed within our bodies.
Antoine Lavoisier, a pioneer oxygen chemist, had pointed out in 1830, that, animals that respire are true combustible bodies, that burn and consume themselves (Lehninger et al., 1990). The biological combustion produces harmful intermediates called free radicals.

Free radicals are fundamental to any biochemical process and represent an essential part of aerobic life and our metabolism. They are continuously produced by the body's normal use of oxygen such as respiration and some cell-mediated immune functions. These free radicals are also generated through environmental pollutants, cigarette smoke, automobile exhaust fumes, radiation, air pollutants, pesticides, etc. (Tiwari, 2001). These exogenous pollutants generating free radicals, have become part and parcel of our daily inhaling / ingesting life and in fact there appears no escape from them. Continuous interaction of the animal physiological systems with these free radicals generated either indigenously or inhaled / ingested from exogenous sources therefore, lead to excess load of free radicals and cause cumulative damage of protein, lipid, DNA, carbohydrates and membrane, resulting in so-called oxidative stress. Therefore, living creatures have evolved a highly complicated defense system with antioxidants composed of enzymes and vitamins against oxidative stress in the course of their evolution. These defense systems are mainly classified (Noguchi et al., 2000) as (i) suppression of generation of reactive oxygen species (ROS), (ii) scavenging of ROS, (iii) clearance, repairing and reconstitution of damage
and (iv) induction of antioxidant proteins and enzymes.

The definition for antioxidants has been extended to any substance that when present at low concentrations compared to those of an oxidizable substrate significantly delays or prevents oxidation of that substrate like lipids, proteins, DNA and carbohydrates (Halliwell, 1990). Currently however, biological antioxidants have further assumed a broad definition to include repair systems such as iron transport proteins (e.g. transferrin, albumin, ferritin and caeruloplasmin), antioxidant enzymes and factors affecting vascular homeostasis, signal transduction and gene expression (Frankel and Meyer, 2000).

An antioxidant works by retarding the oxidation. In biology, oxidation is often started by free radicals. The role of an antioxidant is to intercept a free radical before it can react with the substrate.

However, amounts of these protective devices present under normal physiological conditions are sufficient only to cope with the normal threshold of physiological rate of free-radical generation. Therefore, any additional burden of free radicals, either from an indigenous or exogenous source on the animal (human) physiological system can tip free radical (prooxidant) and anti-free radical (antioxidant) balance leading to oxidative stress (Tiwari, 2001). The oxidative stress, defined as the imbalance between oxidants and
antioxidants in favor of the former, potentially leading to damage has been suggested to be the cause of aging and various human diseases (Sies, 1982). Therefore, in modern Western medicine, the balance between antioxidation and oxidation is believed to be a critical concept for maintaining a healthy biological system (Davies, 2000; Finkel, 2000). Any vitiation therefore, is understood to give rise to disorderliness in the physiological system leading to a variety of diseases depending upon the sensitivity and susceptibility of the organ. Thus, the status of protective mechanism against oxidants, the antioxidants in humans, reflect the dynamic balance between antioxidant defense and prooxidant conditions and have been suggested as a useful tool in estimating the risk of oxidative damage (Papas, 1996; Nose, 2000; Polidori, 2001; Tiwari, 2001).

The cellular antioxidant defenses against both reactive nitrogen and oxygen, containing species are scavengers, both enzymatic (Catalase, Glutathione peroxidase, haeme oxygenase) and non-enzymatic (Glutathione, uric acid, ascorbic acid, α-tocopherol). However, when generation of reactive species exceeds the capacity of endogenous scavengers to neutralize them, tissues become vulnerable to damage. Most tissues can tolerate mild oxidative stress, which often leads to the induction of defense systems, such as antioxidants, metallothioneins and heat shock proteins. However, severe oxidative stress disrupts cell metabolism and can lead to cell death, causing damage to DNA, lipids and proteins.
Antioxidants are an integral part of our defenses against endogenous mutagens. There is abundant evidence in vivo that antioxidants can protect (Odagiri et al., 1992; El-Nahas, et al., 1993) and that depletion of antioxidants can sensitize (Tyrrell and Pidoux, 1986) to DNA damage. Nevertheless it is disconcerting if these compounds themselves can demonstrate DNA-damaging activity.

Specific enzymes such as SOD, Catalase and Glutathione peroxidase clearly constitute an important component of protection against reactive oxygen species, but they do not necessarily protect completely on their own. Endogenous scavengers, such as Glutathione, when present extracellularly, can induce DNA damage in intact lymphocytes, as well as in a cell-free system.

During the 1960s to 1970s, the pioneering work of Meister and his colleagues both clarified the enzymatic basis of the rather unusual mode of peptide synthesis employed, and as well provided a metabolic basis for several heritable diseases resulting from dysfunction in some of these enzymes. It was subsequently proposed that this family of enzymes served as a front-line defense against electrophilic insult. Today, this function of GSH is placed centrally in concepts such as drug and foreign compound detoxification and multi-drug resistance [(Boyland and Chasseand, 1969; Smith, 1977; Meister and Anderson, 1984; Moscow and Dixon, 1993)]
It was during the 1980s, however, that the antioxidant function of GSH attracted particular attention in toxicological research, particularly in relation to the mechanisms of toxicity of both redox-cycling drugs and foreign chemicals [(Moldeus and Orrenius, 1984) cf: Cotgreave and Gerdes, 1998]. Such studies led, by the mid 1980s (Sies and Cadenas, 1985), to a clear biochemical and cytological definition of the concept of "oxidative stress". This concept defines a dynamic situation in which, the balance between the occurrence of oxidants and antioxidants in biological systems, can indirectly influence cellular phenotype, through an effect on other cellular components, particularly redox-sensitive functional groups on proteins.

Pioneering work in the mid 1980s by Thomas and his co-workers, showed that Glutathione may interact in a reversible manner with protein cysteiny1 thiols of many cellular proteins during bouts of oxidative stress [(Grimm et al., 1985) cf: Cotgreave and Gerdes, 1998].

The demonstration of enzymes capable of catalyzing protein thiol-disulphide interchange involving GSH, began to suggest that GSH may have an important role to play in the regulation of complex biochemical processes in intact cells. Examples of such catalysts include Glutathione reductase [(Bellomo et al., 1987) cf: Cotgreave and Gerdes, 1998], the Thioredoxin
(TRX), Thioredoxin reductase (TRX red) system and protein-disulphide isomerase (PDI) (Freedman et al., 1995).

During the 1980s, most focus was placed on the detrimental effects of oxidants in biological systems, particularly the role of oxidative stress in necrotic cytotoxicity. However, in 1985, Cerutti postulated that the occurrence of oxidative stress in cells had profound stimulatory effects on cell proliferation in the process of tumor promotion [(Cerutti, 1985) cf: Cotgreave and Gerdes, 1998].

Several studies have shown that both H$_2$O$_2$ and O$_2^-$, added exogenously to a variety of mammalian cells have mitogenic effects. Some evidence also suggests that the response of tumor cells is particularly amenable to oxidant activation. A number of studies have concentrated on the proliferative index of cells in response levels of reactive oxygen metabolites generated endogenously in cells, either as a consequence of endogenous metabolism or the metabolism of prooxidant compounds. Thus, addition of antioxidant principles to cells, such as SOD or Catalase have been shown to have anti-proliferative effects in cells, presumably by lowering endogenous levels of oxidants in the cells [(Burdon, 1989; Burdon, 1990; Burdon and Gill, 1993; Fiorani et al., 1995) cf: Cotgreave and Gerdes, 1998].

Some of the earliest concrete ties between apoptosis and oxidative stress
were achieved by demonstrating that \( \text{H}_2\text{O}_2 \) induces apoptosis in a number of different cell types, particularly of lymphocytic origin (Sandstrom et al., 1993). Since these early beginnings it has become apparent that a number of prooxidant and other agents also induce apoptosis in cells by elevating the intracellular concentration of reactive oxygen metabolites.

Recently, Loo (2003) has reviewed redox-sensitive mechanisms of phytochemicals (particularly antioxidant polyphenols) mediated inhibition of cancer cell proliferation. Cancer cells, particularly those that are highly invasive or metastatic, require certain level of oxidative stress to maintain a balance between undergoing either proliferation or apoptosis. They constitutively generate large but tolerable amounts of \( \text{H}_2\text{O}_2 \) that apparently function as signaling molecules in mitogen-activated protein kinase pathway to constantly activate redox-sensitive transcription factors and responsive genes that are involved in survival of cancer cells as well as their proliferation. With such a reliance of cancer cells on \( \text{H}_2\text{O}_2 \), it follows that if the excess \( \text{H}_2\text{O}_2 \) can be scavenged by phenolic phytochemicals having antioxidant activity, the oxidative stress-responsive genes can be suppressed and consequently proliferation of cancer cells can be inhibited.

In addition to a correlation between the occurrence of elevated oxidative levels in cells and the tendency to undergo apoptosis and oxidative stress come from the demonstration that a wide variety of antioxidants function as
anti-apoptotic agents against a wide variety of stimuli in different cell types.

Strong evidence has accumulated that, alterations to the intrinsic intracellular antioxidant capacity greatly affects apoptotic responses. The levels of intracellular GSH are very important for the correct execution of apoptotic programming. GSH, which is strictly conserved throughout all higher forms of aerobic life, indeed, plays a role in controlling mammalian cell proliferation, both at the levels of mitogenesis and apoptosis.