5.1 Mitomycin C and Cisplatin

The chemotherapeutic drugs Mitomycin C and Cisplatin employed in the experiments were chemicals with properties of known clastogenecity. In the present work, it was observed that these two test chemicals had induced significant level of genotoxicity (P< 0.001) in CA assay (Table 2 and 5), MN frequency (Table 3 and 6) and SHA assay (Table 4 and 7) studied after 24 and 48 hour (in the case of CA and MN study) and after 35 days (in the case of SHA) of exposure when compared to untreated control groups.

Mitomycin C which has been efficiently used in the treatment of various cancer like gastric cancer, pancreatic cancer, breast cancer, non-small cell lung cancer, cervical cancer, prostate cancer and bladder cancer (Verweij and Pinedo, 1990) was stated to possess a quinone chemical structure which through a cascade of bio-reductive process generates OH· radical of high reactivity which was considered to have potential to directly damage the DNA (Gutierrez, 2000; Korkina et. al., 2000) as well as other biomolecules of cell. Since, free radicals are highly reactive and termed as reactive oxygen species
(ROS), they are prone to undergo reduction by oxidation of surrounding molecules (DNA, lipids, proteins). Oxidation (loss of electrons) and reduction (gain of electrons) are always coupled, and are termed as redox reactions and the damage brought to the biological molecules by such reactions leads to numerous pathological disorders including aging and cancer (Ames et. al., 1983; Feig et. al., 1994). The major harmful ROS responsible for oxidative DNA damage in cells appears to be hydroxyl radicals (OH\(^{-}\)) generated by various reactions of hydrogen peroxide (H\(_{2}\)O\(_{2}\)). The hydrogen peroxide can be produced from the superoxide radical anion by either spontaneous or superoxide dismutase (SOD) mediated disproportionation. The hydrogen peroxide on reaction with the transition metal ions (Henle and Linn, 1997) like iron etc. gives rise to hydroxyl ion or superoxide radical. Such reactions are commonly known as the fenton reactions. Few reactions which can illustrate the mechanism of production of hydroxyl radical and superoxide radical are as follows:

\[
\begin{align*}
O_2 & \xrightarrow{e^-} O_2^- \\
& \xrightarrow{2H^+} H_2O_2 \\
& \xrightarrow{e^-} \cdot OH + OH^- \\
H_2O_2 + Fe(II) & \rightarrow HO^- + OH^- + Fe(III) \\
H_2O_2 + Fe(III) & \rightarrow O_2^- + Fe(II) + 2H^+
\end{align*}
\]

**Figure 44:** Free radical formation mechanism

Other species may also contribute to DNA damage, including metal–peroxide complexes, singlet oxygen, and peroxynitrite (Cadet et. al., 2003;
Since Mitomycin C is a bioreductive alkylating agent, it also damages DNA by cross-linking bases in the same or adjacent strands of DNA principally at the N2 position of the guanine (G) forming monofunctionally and biofunctionally alkylated G-MMC monoadducts and G-MMC-G interstrand and intrastrand cross-links at CpG and GpG sites, respectively (Warren, and Co-workers, 2001) which may eventually lead to apoptotic cell death (Fritsche et al., 1993). Various DNA-adducts formed by Mitomycin C has been identified and isolated in different cell types (one example is given in Figure 45). Mitomycin C has also been reported to activate caspase-3, caspase-8 and caspase-9 mediated apoptosis as well as necrosis (Wu et al., 2008).

**Figure 45:** The structure of a cross-link between Mitomycin C and DNA

Cisplatin, also called Cisplatinum and Cisdiamminedichlorido-platinum(II), is a platinum-based cancer chemotherapeutic drug, which has been used in the treatment of various types of cancers, including carcinomas, sarcomas, lymphomas and some germ cell tumors, (e.g., seminomas and germinomas). Cisplatin was the first member of a platinum-based class of therapeutic agents, which now has other members too. It has the powerful property of being an
antitumor agent and exerts its effect via interaction with DNA to produce cross-linked DNA adducts that activate checkpoint signaling pathways and thereby induce apoptosis (Kaminski et al., 2008). As early as 1986, East-man suggested DNA to be the major target of Cisplatin. Pinto and Lippard in 1985 forwarded the concept that Cisplatin causes inhibition of DNA synthesis by acting on DNA template rather then on DNA polymerase. The Cis configuration of Cisplatin favors the formation of intra-strand crosslinks in DNA (Roberts and Pascoe, 1972; Roos and Arnold, 1977). Upon entering the cell, Cisplatin is spontaneously hydrolyzed to a strongly electrophilic, bifunctional agent that platinates DNA through the positions occupied by chlorine atoms to the N7 position of deoxyguanosine and deoxyadenosine (Pinto and Lippard, 1985).

![Figure 46:](image)

The major intrastrand dinucleotide DNA adducts [between two adjacent guanine (A) and between adjacent adenine and guanine (B)] formed by Cisplatin.

The major DNA adducts are the intrastrand cross-links that form between two adjacent guanines and between an adjacent adenine and Guanine (Figure 46). In addition, small quantities of monofunctional adduct, DNA-protein cross-links and DNA-interstrand cross-links are also produced (Li and Heflich, 1991). Free
5.2 Role of Vitamin C with Chemotherapeutic drugs Mitomycin C and Cisplatin: Mazumdar, 2010

Radicals formation may be another important mechanism in the development of Cisplatin induced toxicity (Masuda et al., 1994; Baliga et al., 1994). It is considered and accepted that the toxicity of the drug Cisplatin is contributed by the platinum metal present in its structure.

There are many evidences where it had been shown that general drugs other than the anti-cancer drugs used in the treatment of various ailments were found to cause damage to the somatic cells as well as germ cells. As for example, Maura and Pino (1991) had reported that Norfloxacin might be responsible for induction of sperm abnormality in mice. Hence most drugs be it an anticancer drug or any other variety, it is found to generate genotoxic and cytotoxic side-effects to a great extent.

5.2 Role of Vitamin C with Chemotherapeutic drugs Mitomycin C and Cisplatin:

Vitamin C is a powerful antioxidant that acts as a free radical scavenger. The role of Vitamin C against oxidative DNA damage is a matter of much controversy (Rivière et al., 2006). Innumerable studies point to the protective effect of Vitamin C supplements (Ajey et al., 1992; Sardas et al., 2006; Demirba et al., 2006; Harapanhalli et al., 1996; Song et al., 2006), whereas several studies had exhibited short-term effects, no effect, or even prooxidant effect of Vitamin C (Moerte et al., 1985; Audera et al., 2001).
Bone marrow cells and germ cells are susceptible to oxidative damage and sensitive to clastogenic chemicals. In the Vitamin C supplementation study, it was experimented to find out whether Vitamin C had any protective role in the Mitomycin C and Cisplatin induced genotoxicity in the somatic as well as germinal cells in mice. In the experiments conducted with Vitamin C, it was observed that the genetic damage induced in the bone marrow cells by Mitomycin C was reduced by prior administration of the antioxidant Vitamin C to a very significant level (P< 0.001) in a dose dependent manner with the highest dose (V3) exhibiting highest level of protection in both 24 and 48 hour studies (Table 2, 3, 4 and Figure 12, 13, 14). This clearly indicates that Vitamin C has the capacity to modulate the oxidative damage induced in mammalian bone marrow cells by some mechanism. Reports support that antioxidant supplements increase the resistance of lymphocytes to oxidative damage (Sardas et al., 2006). Since Vitamin C is a potent reducing agent, it is capable of donating electron to Mitomycin C generated free radical and stabilizes the free radical protecting the chromosomes from the harmful reactivity of ROS. Vitamin C donates two electrons from a double bond between the second and third carbons of the 6-carbon molecule. In the process Vitamin C may gets transformed into semihydroascorbate radical or ascorbyl radical. As compared to other free radical ascorbyl radical is relatively more stable with a half life of $10^{-5}$ seconds and fairly unreactive. This property explains the essence of Vitamin C being a powerful antioxidant. This ascorbyl radical with its unpaired...
electron is further capable of donating a second electron to another ROS and getting transformed to a dehydroascorbate radical which is considered to be less reactive and gets degraded in the process by hydrolysis (Figure 47). There are also metabolic pathways which can recycle the products back to the substrate form using a NADH/GSH as source of reducing agent (Halliwell, 2001; Padayatty et. al., 2003).

Figure 47: Vitamin C (I) and its mono anion (II), stable ascorbyl radical (III) and dehydroVitamin C (IV).

One research work which strongly support the present finding, is that Vitamin C decreased the frequency of sister-chromatid exchanges induced by the drug Mitomycin C and Cyclophosphamide (Krishna et. al., 1986). Similar protection was observed in rat bone marrow cells treated with Vitamin C or olive oil in combination with the antitumoral drug Doxorubicin (Antunes and Takahashi, 1998).

Similarly in our studies, it was observed that Vitamin C supplementation with Cisplatin, reduced the CAs, MN frequency and SHA induced by Cisplatin alone in the bone marrow cells and testis of mice greatly to a significant level.
Role of Vitamin C with chemotherapeutic drugs Mitomycin C and Cisplatin. Mazumdar, 2010

(P< 0.001), mostly in the highest doses (Table 5, 6, 7 and Figure 15, 16, 17). Hence it is justified that Vitamin C has always been an antioxidant of immense importance in chemotherapy since ages. To further support the present finding, some of the important previous works can be cited here. In one work with wistar rat bone marrow cells, it was reported that treatment with Vitamin C inhibited Cisplatin induced chromosomal damage by about 41% and 70% when compared to the animals treated with Cisplatin alone (Antunes et. al., 2000). Previous, to Antunes et. al., Giri and Co-workers (1998), too reported the protective role of Vitamin C on Cisplatin induced mutagenicity in albino mice. So also in human peripheral lymphocytes, it was shown that Cisplatin induced CAs in vitro, was reduced by Vitamin C to a statistical significant level (Nefic, 2001). Genotoxicity induced by some common pharmaceutical like norfloxacin, a fluroquinolones which also acts by the mechanism of generating free radical like the anti-cancer drugs, could also be minimized by combination therapy with Vitamin C (Alba et. al., 2008).

Vitamin C not only scavenges free radicals but it is also very powerful in activating other endogenous antioxidants (Kutsky, 1973) and also regenerating other antioxidants, including Vitamin E (Chan, 1993). In the MN study which was in accordance with the CA results, it was observed that all the three doses of Vitamin C reduced the frequency of MN to a statistically significant level in the highest doses (P< 0.001) at both 24 and 48 hour in case of both the test chemicals, Mitomycin C and Cisplatin (Table 3, 6 and Figure 13, 16). The
capacity of donating electrons to free radicals, activating and regenerating other antioxidants and increasing resistance of toxin-susceptible cells may be confounding factors behind the antioxidative essence of Vitamin C.

Study of germ cells abnormality is of prime importance since such study indicates the possibility of transfer of faulty genetic information from one generation to next through abnormal gamete cells. It is a parameter of immense importance because of its sensitivity and reliability to identify germ cell mutagens (Wyrobek and Bruce, 1978). The normal head shape of mouse sperm is very distinctive and any changes or alterations brought to it can be easily identified. Generation of abnormal sperms is assumed to be the result of spontaneously occurring errors in the differentiation process, or the consequence of an abnormal chromosome complement (Bruce et al., 1980) but such spontaneous changes in morphology is very low under normal conditions and only under harmful chemical exposure or any other oxidative stress the frequency of aberrant sperms may dramatically rise. Infact, Wyrobek and Bruce (1975), had suggested that abnormality in the sperm morphology is the consequence of CAs brought about by exposure to certain chemicals and they studied the effects of 25 different chemicals which elevated abnormal sperms from the control value 1.2 to 3.4%. Since, the present study was conducted after 35 days of exposure to the test chemicals it clearly indicates that such late changes appearing in sperm morphology was due to changes in
the genes responsible for spermatogenesis and not directly on the mature sperms.

In the present study, both Mitomycin C and Cisplatin induced significant amount (P< 0.001) of SHA after 35 days of exposure to the chemicals compared to the control (Table 4, 7 and Figure 14, 17). These could be due to the induction of point mutations in the early spermatocytes and spermatogonia at the premeiotic stages of spermatogenesis (Hugenholtz and Bruce, 1983). These premeiotic stages of sperms take 35 days to complete the process of spermatogenesis from primordial germ cells into mature sperms. Since genes which are responsible for controlling the sperm head shapes are contained in the autosomes and the drugs employed in the experiments probably had caused certain alterations in the testicular DNA which had resulted into sperms with abnormal heads. However, the actual mechanism behind induction of sperm DNA alterations is not clearly understood though innumerable theoretical and practical based assumptions are available. The possibilities may consist of oxidative stress, abnormal chromatin packing and aborted apoptosis (Sharma, et. al., 2004). There may also be the possibility of the test chemicals interfering and causing hindrances with the differentiation process of sperm development (Rai and Vijayalaxmi, 2001), bringing small alterations in the testis DNA (Topham, 1980).

The three doses of Vitamin C (V1, V2 and V3) reduced the frequency of aberrant sperms induced by Mitomycin C, which was found to be statistically
significant (P< 0.001) in the case of middle and high doses (Table 4; Figure 14). So, also experiments with Cisplatin and Vitamin C showed level of protection in the higher doses as well (Table 7; Figure 17). This implies that Vitamin C has a protective role to play in a dose dependent manner against the germinal DNA toxicity induced by Mitomycin C and Cisplatin.

Vitamin C was reported to protect sperm DNA from the damage induced by exogenous oxidative stress in vitro (Song et. al., 2006). It was stated by some workers that a correlation exists between Vitamin C and sperm DNA integrity. It was found that concurrent administration of Vitamin C to pesticide fed animals ameliorates the induced sperm morphology and significantly improves the sperm count (Khan and Sinha, 1996). It was also reported that Vitamin C pretreatment prevented hydrogen peroxide induced sperm DNA damage (Donnelly et. al., 1999). Rolf et. al., (1999) and Greco et. al., (2005) reported that treatment with Vitamin C and E significantly reduced sperm DNA damage. Most recently, it was shown that there is a direct correlation between Vitamin C and fertility in man (Colagar and Marzony, 2009). It was observed that there were high levels of Vitamin C in the seminal fluid of fertile man compared to the amount present in the infertile man since Vitamin C was positively correlated with the percentage of sperms with normal morphology. The role of Vitamin C in the physiology of testis is associated with protein metabolism (Levine, 1986). Enzymatic functions of Vitamin C are essential for
the normal integrity and function of testis i.e. synthesis, maintenance and development of normal sperms (Dawson et al., 1990).

The above mentioned reports are in agreement with our present findings of Vitamin C significantly reducing the SHA. The protective effect observed in the present study may be attributed to the antioxidant and free radical scavenging properties of Vitamin C (Miller, 1990; Duthie et al., 1996; Sardas, 2006).

5.3 Role of Vitamin E with Chemotherapeutic drugs Mitomycin C and Cisplatin:

Vitamin E, the most potent lipid peroxyl radical scavenger has a profound significance of being an antioxidant, (Traber and Atkinson, 2007) a neuroprotector (Khanna et al., 2005; Khanna et al., 2006) a protector against atherosclerosis, carcinogenesis (Coulter et al., 2006; Dutta and Dutta, 2003), cardiovascular disease (Shekelle et al., 2004) and also having strong anti-inflammatory properties (Reiter et al., 2007). Inspite of innumerable works, some which reports it to be a potent antioxidant, some which report it to be a prooxidant and some which reports no marked effect of this chemical in terms of its antioxidative properties are found to be very confusing and inconclusive. So also the data and literature available in this regard are highly contradictory. Hence further research needs to be conducted to determine the dietary levels of Vitamin E in order to protect the gene pool from endogenously or exogenously induced DNA damage (Claycombe and Meydani, 2001).
In the present work, it was observed that lone treatment with Vitamin E delivers significant genotoxicity in the middle (VE2) and high (VE3) dose of Vitamin E which included mostly breaks and gaps (Table 8, Figure 18). In case of MN test too, the middle (VE2) and high (VE3) doses of Vitamin E exhibited significant level of MN frequency (Table 9, Figure 19). This provide the hint that that there are various aspects to the biochemistry and behavior of Vitamin E which in addition to its anti-oxidative properties may also have the attribute of genotoxicity based on the doses employed.

It was quite interesting to observe in the present study that doses of Vitamin E which was found to be genotoxic when used alone but when employed prior to the drugs Mitomycin C and Cisplatin, it showed protective role against the drug induced genotoxicity. Hence in the Vitamin E supplementation studies with Mitomycin C and Cisplatin it was observed that there was a significant decline in the level of chromosomal damage (Table 8 and 11), MN frequency (Table 9 and 12) and SHA (Table 10 and 13) mostly at the higher doses (P< 0.001). This indicates that there is a dose dependent suppression of mutagenecity by Vitamin E on the oxidative stress induced by Mitomycin C and Cisplatin. It is very difficult to justify such findings. However, considering the biochemistry and behavior of both this chemicals, it can be assumed that Vitamin E when acting alone releases peroxyl radicals and these radicals are capable of inducing genetic damage in absence of sufficient and proper amount of radical stabilizing counterparts. However, when both this agents were
subsequently administered one after another, there is a probability that radicals generation could be inhibited as one counterpart is capable of stabilizing the other counterpart through redox reactions. A dose-ranging studies in human participants supplemented with different doses of Vitamin E was conducted (Jackson et al., 2007). The study provided information on the dosage of Vitamin E that decreases systemic oxidant stress in a linear trend with the maximum reduction at the higher doses. Hence in the present study, it was observed that there was also dose dependent linear response of Vitamin E with the highest dose (VE3) providing maximum protection (P< 0.001).

Alpha-tocopherol is the best antioxidants amongst all the Vitamin E forms. It had exhibited protection against various toxic elements in both in vivo and in vitro studies. Vitamin E's function as a protectant against genotoxicity is dependent upon its ability to break radical-propagated chain reactions. As a result, the formation of the tocopheroxyl radical, the odd-electron derivative of Vitamin E, is an inherent part of any Vitamin E based, antioxidative reaction (Ingold et al., 1987). As a matter of fact, lipid-soluble antioxidant, alpha-tocopherol in biological membranes, reacts with many oxidant molecules. It infact helps in protection of cell membranes from lipid peroxidation by trapping peroxyl radicals. It involves the abstraction of a hydrogen atom from the OH group of the tocopherol by a peroxyl (oxidant) molecule. Upon the formation of the tocopheroxyl radical from a reaction between Vitamin E and an oxidant molecule, the radical formed is free to interact with another peroxyl radical. This
reaction generates a stable tocopheroxyl radical which do not further propagate radical chains. This, tocopheroxyl radical can be regenerated back to alpha-tocopherol by an electron donor, like Vitamin C, and is thereby able to maintain cellular antioxidant protection over a period of time (Dutta et. al., 2003). This mechanism can be conceptualized to be involved in neutralizing the free radicals generated by the test drugs employed and minimizing its genotoxic effects on the DNA of cells.

Vitamin E (20 mg/kg) at 24 hour pretreatment to mice delivered 100% protection to the lethal dose of iron and when given within 5 to 60 minutes of acute iron intoxication reduced mortality to nearly 75% (Omara and Blakeley, 1993). Oxidative stress may be involved in chronic renal failure. To illustrate this and to study the role of Vitamin E supplementation in this aspect, Kan et. al., (2002), established that Vitamin E supplementation visibly protected DNA strand breaks in the lymphocytes of dialysis patients after 14 weeks of therapy which indicates that it is capable of neutralizing the oxidants involved in DNA strand breaks.

In one work, application of Vitamin E ameliorated the chromium and or nickel induced oxidative stress in the mouse liver. It prevented lipid peroxidation as well as protected the antioxidative system (Rao et. al., 2006). In another previous work, it was demonstrated that Vitamin E was capable delivering protection from lipid peroxidation, ionizing radiation and oxidative DNA damage on the human HCC cell line (Fantappie et. al., 2004). Vitamin E supplementation in
patients treated with Cisplatin Chemotherapy was found to deliver neuroprotection to Cisplatin induced peripheral neurotoxicity without affecting the drug efficacy (Pace et. al., 2003). Vitamin E in dose (400 mg/kg/bw) along with Vitamin C and Vitamin A was found to inhibit Dimethylaminoazobenzene (DAB) induced hepatoma in male albino rats which clearly suggests that Vitamins are capable of preventing oxidative damage associated with cellular damage (Velanganni and Balasundaram, 2003).

In the present work, Vitamin E supplementation studies with Mitomycin C and Cisplatin clearly show that Vitamin E resulted in very high level of protection to sperm head against drug induced toxicity and the highest doses reduced the frequency of SHA to almost 50% when compared to only the drug treated groups (Figure 20 and 23). This generates the idea that Vitamin E is very potent against the genotoxicity induced by those drugs through various mechanisms as stated earlier. ROS being highly active oxidants and being generated by these drugs may affect the developing spermatozoa. Vitamin E has been reported to inhibit lipid peroxidation of polyunsaturated fatty acids because of its chain breaking properties (Wen, 2006). It was stated in one work that intake of lipid soluble antioxidants like alpha-tocopherol protects against the oxidative DNA damage caused by high PUFA diet (Jenkinson et. al., 1999).

In human it was clearly and strongly demonstrated that supplementation with Vitamin E and Vitamin C in infertile males can improve semen quality and fertility in patients (Nouri et. al., 2008).
5.4 Role of Quercetin with Chemotherapeutic drugs Mitomycin C and Cisplatin:

Quercetin, the most potent of the plant flavonoids, was found to induce significant levels of genotoxicity in the present work, mostly of which included breaks and CAs like sticky chromosomes and C-mitosis in all the doses applied (Table 14, Figure 24 and 25). In the case of MN test too, the doses exhibited a significant level of MN frequency (Table 15, Figure 26). So also in the case SHA studies it was observed that with the increase in Quercetin doses there was a gradual and significant rise in the percent aberrant sperms compared to the untreated control (Table 16, Figure 27). This clearly indicates that Quercetin has got a mutagenic role to play.

However, in the combination studies, when Quercetin was supplemented prior to Mitomycin C treatment it exhibited a protective role minimizing the chromosomal damage to a significant level at the higher doses (P< 0.001) (Table 14). Again, when it was supplemented prior to Cisplatin administration it did not show any level of protection in all the three mutagenecity tests (Table 17, 18, 19 and Figure 28, 30, 31). So also the incidence of C-mitosis and sticky chromosomes were observed in combination treatment of Quercetin and Cisplatin (Figure 29). Therefore it appears that there exists more than one mechanism by which Quercetin can act to generate DNA damage in certain cases, whereas in some cases, when operating with other chemicals it can
deliver protection against the genotoxicity generated by those chemicals, acting antagonistically against those chemicals and on the other hand working in synergistically with some other chemicals.

The potential health benefits of Quercetin and its varied biological properties had generated a vast era of research on various aspects of its metabolism and biological activity. Most of these studies have been performed either in cell free system or in cells in vitro. Therefore, it is not easy to extrapolate these findings to in vivo situation because of differences in cellular and hepatic metabolism. The prooxidant chemistry of Quercetin is important because it is related to the generation of mutagenic quinone-type metabolites (Boots et al., 2008; Rietjens et al., 2005; Yen et al., 2003).

In the present study, as mentioned above, Quercetin induced dose-dependent increase in the frequency of MN as well as significantly higher frequency of CA in the bone marrow cells of mice. The findings strongly indicate that Quercetin in the dose ranges tested may be considered genotoxic in vivo. These findings are in accordance with earlier studies reporting the genotoxic effect of Quercetin (Silva et al., 2000). The DNA damaging effects of Quercetin had been shown in other studies as well (Yen et al., 2003; Yamashita et al., 1999). Flavonoids with molecular structure similar to Quercetin are reported to cause peroxidation of oxidized nuclear membrane lipids as well as DNA strand breaks in isolated rat liver nuclei through hydroxyl radical production (Sahu and
Gray 1997; Crole et al., 2000). A number of polyphenols, including Quercetin, can bind to DNA and this direct interaction may be an important mechanism of mutageniciry (Alvi et al., 1986). The mechanism of interaction of Quercetin with DNA may be complex and depend on many factors. The intracellular and extracellular environment and presence of metals may additionally complicate the interaction (Crole et al., 2000). Structural requirements for mutagenicity of Quercetin include a flavonoid ring structure with a free hydroxyl group at the 3-position, a double bond at the 2,3-position, and a keto-group at the 4-position allowing the proton of the 3-hydroxyl group to tautomerise to a 3-keto moiety (Omenn, 1997). It was reported that Quercetin gets converted into a potentially toxic product while offering protection by scavenging reactive oxygen species in the cell (Boots et al., 2007). Spencer et al., (2003), studied the intracellular metabolism of Quercetin and found three of its major metabolites to be 3'-O-methyl Quercetin, 4'-O-methyl Quercetin and Quercetin 7-o-β-Dglucuronide in human fibroblast cells. They had found that free Quercetin is metabolized to 2'-glutathionyl Quercetin and Quercetin quinone/quinone methydes. On the other hand, 3'-O-methyl Quercetin because of the presence of a free 4' OH group in the B-ring is able to autoxidize to 3'- O-methyl Quercetin 5-quinone methyde. Quercetin orthoquinones and quinone methydes are reported to be responsible for the prooxidant, alkylating and DNA-reactive property (Boots et al., 2007; Metodiewa et al., 1999; Free Radical Biol. Med.). This observation of intracellular formation of alkylating Quercetin metabolites was corroborated by
results from studies of Walle et. al., (2003), demonstrating the formation of covalent protein and DNA adducts in Caco-2 and HepG2 cells exposed to (14 C)-labeled Quercetin. Doubts have been raised whether the *in vitro* genotoxic results of Quercetin will have any impact *in vivo* considering the reversible nature of the Quercetin methyde adducts and/or the repair of DNA adducts. The present findings suggest the potential of Quercetin to induce genotoxicity *in vivo*. Hydroxyl radicals produced close to the DNA backbone could induce direct site-specific strand breaks that are insensitive to the free radical scavengers. The lack of dose response correlation observed for CA in the present study could be due to the fact that the formation and break down of Quercetin methydes could reach equilibrium at a certain upper threshold of Quercetin concentration in the target cells so that proportionately higher frequency of damage is not seen with increasing the dose. In a study using 20, 200, 400 and 800 mg/kg of Quercetin, Castillo et. al., (1989), showed that the 20 mg/kg dose had significant anti-proliferative effect, which was only slightly less than that the effect seen with 800 mg/kg in mice *in vivo* bearing transplantable tumors. Quercetin is known to be a Topoisomerase II inhibitor/poison resulting in endoreduplication and improper anaphasic movement of chromosomes (Cantero et. al., 2006). Significant increase in the frequency of metaphases with sticky chromosomes as well as occurrence of C-mitosis (Figure 25 and 29) indicates the deleterious effect of Quercetin on chromosome separation and the spindle apparatus. Correlation between spindle aberration
and formation of sticky chromosomes as well as C-mitoses has been reported by Ibrulj and Efendic-Tasevac (2002). Therefore, the observed differences in the dose response correlation for CA and MN formation could be attributed to the different mechanisms of their formation. Further, the existence of separate upper threshold concentrations of Quercetin for induction of CAs and MN formation cannot be ruled out. The higher frequency of CA and MN observed at 48h of treatment could be due to residual effect or relatively longer presence of Quercetin and/or its metabolites in the target cells. The intracellular oxidation products of 3'-O-methyl Quercetin and Quercetin (orthoquinone and quinone methide) are reported to persist up to 18h within the cell indicating they are relatively long-lived species (Spencer et. al., 2003).

Quercetin metabolites are reported to have significantly altered redox potentials (Day and Williamson, 2003). During intracellular metabolism, Quercetin produces GSH adducts where as its metabolites 3'-O-methyl Quercetin and 4'-O-methyl Quercetin do not form any GSH adducts (Spencer et. al., 2003; Awad et. al., 2002) The GSH adduct is generally considered to be cytoprotective (Monks and Lau 1998; Bolton, 2002) only when GSH conjugation is coupled with the export of adducts from cells (Ishikawa, 1992). On the other hand, conjugation with GSH often enhances the redox activity of polyphenols and thus does not necessarily result in detoxification. Higher dose of Quercetin can result in cellular thiol depletion and cause toxicity (Spencer, et. al., 1995). In fact, quinone and semiquinone are known to facilitate super oxide formation.
5.4 Role of Quercetin with chemotherapeutic drugs Mitomycin C and Cisplatin. Mazumdar, 2010

(Metodiewa et. al., 1999). This is further corroborated by the studies of Spencer et. al., (2003) who reported that while Quercetin up to 10 M conferred protection against fibroblast damage induced by oxidative stress, 30 M resulted in toxicity. Pre-treatment of Quercetin reduced MMC induced frequency of MN as well as CA.

However, no clear dose-response correlation could be found. Quercetin has been shown to provide protection against radiation induced DNA damage (Spencer et. al., 2003; Devipriya, et. al., 2008; Jin et. al., 2006) and cadmium (Blasiak, 2001). Blasiak observed stimulation of DNA repair process following Quercetin treatment in cadmium treated lymphocytes and speculated that Quercetin may regenerate DNA repair enzymes in lymphocytes. On the other hand, in another study, Quercetin at 50 μM decreased the level of DNA damage induced by hydrogen peroxide, it did not influence the rate of DNA repair in Caco-2, HepG2 and V79 cells (Aherne and O'Brien, 2000). In yet another study, supplementation of 600g of fruits and vegetables for 24 days failed to alter the expressions of DNA repairing genes oxoguanine glycosylase 1 and excision repair cross-complementing 1 in leukocytes (Moller et. al., 2003).

The mechanism of interaction between Mitomycin C and Quercetin is not known. Therefore, it cannot be ascertained whether the observed decrease in Mitomycin C induced MN and CAs frequency is a phenomenon of attenuation (antioxidant activity and/or stimulation of DNA repairing process) or synergism (enhanced apoptotic process resulting in lower recovery of viable mutants).
Quercetin has been shown to act synergistically with Cisplatin (causes 1.5- to 30-fold potentiation of cytotoxicity) and busulphan (Hofmann et. al., 1990; Hoffman et. al., 1989). Therefore, more research is required to elucidate the mechanism of interaction of Quercetin with DNA as well as with other DNA acting agents like Mitomycin C. In SHA it was observed that Quercetin did not induce any protection against Mitomycin C induced genotoxicity.

In supplementation of Quercetin with Cisplatin it was observed that there was no reduction generated in the incidence of CAs, MN formation and SHA but in fact there was increased rise in the frequency of aberrant cells incase of SHA studies. In the work of Akbas et. al., (2005) the cytotoxic activity of chemotherapeutic agent Topotecan in combination with Quercetin in two human breast cancer cell lines, MCF-7 and MDA-MB-231 was studied and it was observed that Quercetin did not inhibit ROS generation and enhanced the cytotoxicity of Topotecan in both cell types.

Quercetin administration in all the doses did not confer any protection to Mitomycin and Cisplatin induced SHA. It was observed that all the three doses of Quercetin when administered alone induced significant level (P< 0.001) of genotoxicity to the germ cells when compared to untreated control (Table 16, 19 and Figure 27, 31). Infact, in combination treatment with Mitomycin C, it was observed that there was an increase in the percent aberrant sperms with increase in Quercetin doses when compared to only Mitomycin C treated
groups (Table 16, Figure 27) and in the case of Cisplatin treated groups with Quercetin the aberration rate remained almost same with no significant reduction in percent aberrant sperms (Table 19, Figure 31). This indicates that Quercetin is mutagenic to the germ cells. Earlier reports of Rastogi and Levin (1986) has shown that Quercetin treatment to mice have induced SHA to a significant level when compared to control and this finding was very much in agreement with our results.

In conclusion, the precise mechanisms by which flavonoids exert their beneficial or toxic actions are unclear. A major confounding factor is the dose, since there is hardly any consensus among various studies regarding the dose at which Quercetin may exert its beneficial and/or toxic effects in vivo. Therefore, a clear understanding of the mechanism of action of Quercetin with respect to its primary cellular site of action, dose and interaction with other bioactive molecules in model systems that respect the dynamics and integrity of complex biological networks in vivo is important to evaluate potential of Quercetin as a chemo-preventive and/or therapeutic agent. The present findings suggest that Quercetin has the potential to produce genotoxic effects in vivo, which should be taken into consideration in further studies while evaluating its possible use as a therapeutic agent in vivo, which should be taken into consideration in further studies while evaluating its possible use as a therapeutic agent.
5.5 Role of Curcumin with Chemotherapeutic drugs Mitomycin C and Cisplatin:

Curcumin had exhibited anticlastogenic activities in innumerable in vivo and in vitro studies. In the present work, Curcumin exhibited great potential in inhibiting the genotoxicity generated by Mitomycin C and Cisplatin. In the CA study (Table 20, Figure 32) and MN study (Table 21, Figure 33) with Mitomycin C and Curcumin, it was observed that there was a great reduction in the genotoxicity level (P< 0.001) in all the three doses employed in the experiments. In the work with Cisplatin and Curcumin it was observed that there was a gradual decline in CAs with the highest dose of Curcumin delivering significant level (P< 0.001) of protection compared to only Cisplatin treated groups in the 24 hour study group (Table 23) and in the MN study as well it was observed that the frequency of MN declined statistically significant in the middle (P< 0.05) and highest dose (P< 0.01) of Curcumin (Table 24, Figure 36).

Similarly, in the SHA studies with Mitomycin C and Curcumin it was observed that there was a dose dependent decline in the frequency of abnormal sperm in all the three doses of Curcumin (P< 0.001) when compared to only Mitomycin C treated groups (Table 22, Figure 34). So also with Cisplatin and Curcumin treated groups it was observed that there was gradual decline in the frequency of aberrant sperms in middle (P< 0.01) and highest doses (P< 0.001) when compared to only Cisplatin treated groups (Table 25, Figure 37).
The protection delivered by Curcumin to Mitomycin C and Cisplatin induced genotoxicity could be clearly attributed to the antioxidative effects of the polyphenol. There are previous reports where Curcumin had exhibited protection against various chemotherapeutic agents. The work of Mukhapadhaya et al., (1998) directly supports the present work where anticlastogenic property of turmeric and Curcumin was demonstrated against Cyclophosphamide and Mitomycin C induced genotoxicity in vivo.

Other than chemotherapeutic agents, Curcumin has also exhibited protection against metal induced genotoxicity. It was demonstrated that Curcumin generated protection against copper genotoxicity in vivo (Rivera et al., 2007). It was also observed that dietary Curcumin lowers lipid peroxidation induced by iron in the rat liver by enhancing the activities of endogenous antioxidants. Curcumin exerts powerful inhibitory effect against H$_2$O$_2$-induced damage in human keratinocytes and fibroblasts (Phan et al., 2001) and in NG 108-15 cells (Mahakunakorn et al., 2003). Curcumin reduces oxidized proteins in amyloid pathology in Alzheimer transgenic mice. It also decreases lipid peroxidation in rat liver microsomes, erythrocyte membranes and brain homogenates (Pulla Reddy and Lokesh, 1994). This is brought about by maintaining the activities of antioxidant enzymes like superoxide dismutase, catalase and glutathione peroxidase (Pulla Reddy and Lokesh, 1992).
Recently, it was observed that Curcumin prevents oxidative damage during indomethacin-induced gastric lesion not only by blocking inactivation of gastric peroxidase, but also by direct scavenging of $H_2O_2$ and $OH^-$. Since, ROS have been implicated in the development of various pathological conditions, (Bandyopadhyay et al., 1999; Halliwell, 1998; Halliwell and Gutteridge, 1990). Curcumin was found to possess the potential to control those diseases through its potent antioxidant activity. In addition to its antioxidative properties and free radical trapping mechanism, it also forms complex with the mutagen and modulates the mutagen metabolism (Premkumar et al., 2004). Interestingly, Curcumin also enhances the activities of other antioxidative enzymes like glutathione peroxidase, catalase and Superoxide dismutase (Pulla Reddy and Lokesh, 1994). Curcumin was also found to reduce clastogenesis in bone marrow cells of rodents exposed to gamma-radiation or Cisplatin (Abraham et al., 1993; Antunes et al., 2000). Antimutagenic potential of Curcumin was also shown in plant cells where experiments were conducted to find out how Curcumin pre-treatment reduces sodium azide induced CAs in *Allium Cepa* root tip (Irulappan and Natarajan, 2007). Oxidative damage is the major cause behind the pathogenesis of Alzheimer's disease and it had been experimentally proven that Curcumin helps in the control of this disease because of its potent antioxidative capabilities. The major reason behind Curcumin generating such significant level of protection could be attributed to the fact that it is an oxygen free radical scavenger (Kunchandy and Rao, 1990; Ruby et al., 1995; Subramanian et al., 1994; Joe and Lokesh, 1994; Unnikrishnan and Rao, 1995;
Song et al., 2001). Venkatesan and Rao (2000) had established structural relationship between synthetic Curcumin analogues and inhibition of lipid peroxidation and scavenging of free radicals. In all the models, it was shown that the phenolic analogues were more active than the non-phenolic analogues. Hence the antioxidative property of Curcumin is attributed to the phenolic group present in its structure. It was theoretically elucidated that hydrogen abstraction from the phenolic group and not from the central CH$_2$ group in the heptadienone link is involved in the antioxidative mechanism of Curcumin (Sun et al., 2002).

The antioxidative properties of Curcumin can be attributed to its structure where the presence of the phenolic hydroxyl and methoxyl groups present in the phenyl ring and the 1,3 diketone functional groups are responsible for scavenging and neutralizing free radicals by interacting with the oxidative chain reactions (Jovanovic et al., 2001; Sun et al., 2002; Anto et al., 2002). In this study the precise mechanism of protective action by Curcumin is not known and there is a possibility that it must have generated protection to Mitomycin C and Cisplatin induced genotoxicity by one of the above mentioned mechanism.

5.6 Comparative effects of Vitamin C, Vitamin E, Quercetin and Curcumin on Mitomycin C and Cisplatin induced genotoxicity.

In the present work when a comparative study of the antioxidants (Vitamin C, Vitamin E, Quercetin and Curcumin) with the chemotherapeutic drugs Mitomycin C and Cisplatin was done. It was observed that different antioxidants had different types of reactivity with the different drug. It is known
that reactivity of any class of chemical depends on large number of confounding factors, like the endogenous and exogenous cellular environment of the organism, presence or absence of metal ions, body physiology and reactivity to chemicals etc. Here, the drugs employed in the experiments have different chemical and physical properties. They are also specified against different types of cancer and their sensitivity and mode of toxicity generation in living system is quite different from one another. The major chemical difference between the two drug is that Mitomycin C is an alkylating agent whereas, Cisplatin is an chemical whose toxicity is basically generated by the presence of the metal platinum present in its structure.

It was observed that with Mitomycin C the highest level of protection was conferred by Curcumin, followed by Vitamin C, Vitamin E and finally Quercetin in all the three cytogenetical endpoints studied (Table 26, 27, 28 and Figure 38, 39, 40). Curcumin has a long history as a traditional herbal medicine in India and most clinical trials have showed no significant toxicity by Curcumin. In the present study, Curcumin had shown better protection over all the other antioxidants, probably due to the fact that it has a greater half life then Vitamin C and Vitamin E. So, also due to the presence of a unique conjugated structure, which includes methoxylated phenols and an enol form of beta-diketone, a structure which typically represents radical trapping abilities (Chattopadhyay et. al., 2004) it may be attributed of being a potent antioxidant over Vitamin C and Vitamin E which lacks such a unique chemical structure. Moreover it has the
property to stimulate other endogenous antioxidants under oxidative stress which increases its efficiency as an antioxidant (Pulla Reddy and Lokesh, 1994). So also Vitamin C too possess the property of stimulating and expressing other protective endogenous enzymes like superoxide dismutase, catalase, glutathione S transferase and regenerating them back including antioxidant Vitamin E too (Kutsky, 1973 and Chan 1993). Hence Vitamin C probably was found to be effective in delivering protection to Mitomycin C induced genotoxicity. But due to Vitamin C and Vitamin E's shorter half-life compared to that of Curcumin the impact of the antioxidative effectiveness probably gets reduced slightly when compared to Curcumin's effectiveness. Taking into consideration the significant genotoxicity conferred by Quercetin when applied alone and at the same time appreciable level of protection generated by Quercetin when working with the drug Mitomycin C, the potentiality of Quercetin and its application as an antioxidant is less clear. There exists a thin line between its effectiveness as an antioxidant and its role as a prooxidant. A proper pharmacological dose which is physiologically active and with minimal side-effects and a right treatment duration needs to be worked out for reaping the benefits of such multi-faceted and powerful antioxidant.

Whereas, in the case of Cisplatin induced genotoxicity, antioxidant Vitamin E showed the maximum level of protection, followed by Vitamin C and Curcumin (Table 29, 30, 31 and Figure 41, 42, 43). Quercetin showed no protection against Cisplatin induced genotoxicity. The possession of metal
platinum, itself explains the differential behavior and reactivity of Cisplatin with the antioxidants. Out of the four antioxidants, Quercetin and Curcumin were phenolic compounds. When acting with the alkylating agent, Mitomycin C both the polyphenols were found to exhibit high level of protection against genotoxicity induced by the drug, but in the case of Cisplatin induced genotoxicity, Quercetin did not generate any protection whereas the protection delivered by Curcumin was also not very appreciable when compared to the other counterparts, Vitamin E and Vitamin C. It can be proposed that the presence of the metal platinum in the drug, Cisplatin somehow hinders and reduces the antioxidative efficacy of Quercetin and Curcumin. It is however very difficult to conclude any statement regarding the mechanism of action of these drugs with the given antioxidants unless a proper and molecular based study is conducted in this aspect.