5.1 METRONIDAZOLE

Metronidazole is a nitroimidazole antiinfective drug used mainly in the treatment of infections caused by susceptible organisms, particularly anaerobic bacteria and protozoa. Metronidazole is mutagenic in some bacterial test system (Voogd, 1981). Genotoxicity data on metronidazole in mammalian test system is contradictory (Bendesky et al., 2002). Metronidazole is reported to be mutagenic (Rustia et al., 1972; Dobias, 1980; Reitz et al., 1991; Mudry et al., 1994; Nahas and Ashmawy, 2004; Aguirre et al., 2006). Reports to the contrary also found in the literature (Bost, 1977; Lambert et al., 1979; Hartley-Asp, 1979, 1981). Further, there is insufficient data on the effects of metronidazole on sperm morphology. Development of chromosomal aberrations, micronuclei and sperm head abnormality has been commonly used as sensitive biological indicator in the mutagenic bioassays of a drug. In the present study all these
parameters were studied so as to evaluate the genotoxic potential of metronidazole in mammalian test system.

The mechanism of therapeutic action of metronidazole is thought to involve interference with DNA by a metabolite in which the nitro group of metronidazole has been reduced (Citrin et. al., 2001). Metronidazole contains a nitro-group that must be reduced in order to produce its toxicity against microbial (Ings et. al., 1974; Ludlum et. al., 1988) and mammalian cells (Mohindra and Rauth, 1976). Furthermore, nitro group reduction is considered to be responsible for the mutagenicity and the drug residue formation because, without reduction, there is no binding to DNA (La Russo et. al., 1977). It is activated when reduced through electron donation from ferredoxin or flavodoxin that were themselves reduced by the pyruvate: ferredoxin oxidoreductase (Land et. al., 1997), possibly forming a hydroxylamine (Ings et. al., 1974). This process occurs only under strongly reducing conditions (Samuelson, 1999). Metronidazole is also activated in hypoxic cells of animals and hence it has been applied as a radiosensitizer of human tumors (Roy et. al., 1996).

In the present study metronidazole at human equivalent and lower doses induced a variety of chromosomal aberrations in the bone marrow cells (Table–1). The gaps and breaks were more frequent than other aberrations. Since very large number of gaps are observed in the treated groups than that of the control, they may be induced by the clastogenic effect of metronidazole and can
not be explained as artifacts as was suggested by Buckton and Pike (1964). In an assessment of chemically induced chromosome damage, Gebhart (1977) suggested that the gaps might be associated with chemical mutagenesis. Similar large number of gaps has been reported by Giri et al. (2002a, 2002b, 2002c) in their studies with chemical pesticides.

Significant dose response correlation could be found at 24h of the i.p. treatment with the acute dose 10, 20 and 40 mg/kg of metronidazole (Table–1, Figure–5). It has been reported that chemicals in general produce the highest frequency of aberrations in rodents 24h after single exposure, which roughly coincides with the normal length (22-24 h) of the cell cycle (Schmid, 1973). The chronic highest dose (3X13.4 mg/kg) also induced significant increase in the frequency of chromosome aberrations when compared to untreated control. The results of the present study indicate that metronidazole induces the formation of chromosome aberrations in a dose-dependent manner which is in support of the findings of (Rustia et al., 1972; Dobias, 1980; Reitz et al., 1991; Nahas and Ashmawy, 2004). Activated metronidazole is thought to interact directly with DNA and the resultant complex can no longer function as an effective primer for DNA and RNA polymerases. This is the most widely held explanation of its toxic action on cells (Muller, 1983).

Evaluation of micronuclei frequency has been recommended by the regulatory agencies to be conducted as part of product safety assessment
(Krishna and Hayashi, 2000). To test somatic mutagens in vivo, bone marrow micronuclei assay has been used extensively (Tice et al., 1994; Giri et al., 2002a). Micronuclei are small, extranuclear bodies consisting of acentric fragments of chromosomes or entire chromosomes, which lag behind at anaphase of cell division. After telophase, these fragments may not be included in the nuclei of daughter cells and form single or multiple micronuclei in the cytoplasm. They can be easily recognized in the cytoplasm of immature PCEs (Schmid, 1976).

In the present study also a significant increase in the frequency of micronucleated PCEs, NCEs and NCs was observed following metronidazole treatment (Table–2, Figure–6). Significant correlation between the acute dose and the frequency of micronucleated PCEs at 24 h of the i.p treatments was observed. The chronic dose (3x13.4 mg/kg) also induced significant increase in the frequency of micronuclei when compared to untreated control. However, the occurrence of comparatively lower frequency of micronuclei than chromosome aberrations could be due to the fact that the fate of chromosomal fragments is uncertain so far their segregation in micronuclei is concerned. Apart from that, all fragments do not necessarily form visible Micronucleus (Savage, 1988). The results of the present study are in agreement with the studies reported earlier (Mudry et al., 1994; Nahas and Ashmawy, 2004; Aguirre et al., 2006).
Sperm abnormality assay is a sensitive and reliable endpoint and is widely used to identify germ cell mutagens (Wyrobek and Bruce, 1978; Giri et al., 2002a, 2002b; Nahas and Ashmawy, 2004). Induction of abnormal sperms is presumed to be a result of naturally occurring errors in the differentiation process, or the consequence of an abnormal chromosome complement (Bruce et al., 1980). The characteristics controlling sperm head shape are carried on the autosomes and sperm abnormality test identifies those agents, which cause small alterations in testicular DNA (Topham, 1980).

In the present study, all the three acute doses of metronidazole induced significant increase in the frequency of sperm head abnormality after 3 weeks (21 days) of treatment (Table-7). The highest acute and chronic dose showed significant increase in sperm head abnormality after 24 h of treatment. The exact mechanism of sperm DNA damage is not completely understood but there are several possibilities including oxidative stress, abnormal chromatin packing and aborted apoptosis (Sharma et al., 2004). It is suggested that abnormal sperms may arise from interference of the test substance with the genetically controlled differentiation of the sperm cells (Rai and Vijayalaxmi, 2001), and may be a consequence of small alterations in testicular DNA (Topham, 1980). The observed increase in the frequency of abnormal sperms in the present study following 21 days of the treatment may further support the genotoxic potential of metronidazole. This present finding about the reproductive toxicity with metronidazole is also supported by Nahas and
Ashmawy (2004). However, the significantly higher occurrence of sperms with abnormal head morphology at 24h of the treatment (Table–7) may indicate interaction of metronidazole with cell membrane components causing distortions in a non-genetic manner.

In the vitamin C supplementation study, attempt was made to find out if vitamin C (L-ascorbic acid) played any protective role in the metronidazole treated mice so far as induction of chromosome aberration and micronuclei in the bone marrow cells as well as occurrence of sperms with abnormal head morphology is concerned. Reactive oxygen species (ROS) and oxidative DNA damage are implicated in numerous pathological disorders including aging and cancer (Ames et. al., 1983; Feig et. al., 1994). The main reactive species responsible for oxidative DNA damage in cells appears to be hydroxyl radicals generated by the reaction of reactive transition metal ions (Henle and Linn, 1997). Other species may also contribute to DNA damage, including metal–peroxide complexes, singlet oxygen, and peroxynitrite (Cadet et. al., 2003; Frelon et. al., 2003). Although the majority of oxidative DNA damage is repaired by base excision repair (Cadet et. al., 2000), there is probably a low steady-state level of damage that persists even in the absence of oxidative stress.

Vitamin C is a critical antioxidant that acts as a free radical scavenger and may regenerate other antioxidants, including vitamin E and help in protecting these kind of damaged cells (Chan, 1993). It was established that the
distribution of ascorbic acid and its concentration in the organs was subjected to variations depending on introduction of different drugs (Linster, 2007). The role of vitamin C in protecting against oxidative DNA damage is a matter of much controversy (Rivière et al., 2006). On one hand, numerous studies point to a 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). On the other hand, several studies have suggested short-term effects, no effect, or even a prooxidant effect (Moerte et al., 1985; Audera et al., 2001).

In the present study supplementing the animals prior to treatment with metronidazole significantly (P< 0.001) reduced the frequency of chromosome aberrations in the bone marrow cells (Table–2, Figure–6). Similar level of significant protection could also be observed in the frequency of micronuclei in the bone marrow cells (Table–5, Figure–9). The protection was found to be alike across the cell types studied. In the sperm head abnormality assay also vitamin C conferred marked protection both at 24h and 21 days of the treatment (Table–8, Figure–12).

Ascorbic acid, a major water-soluble antioxidant, has recently been reported to protect sperm DNA from the damage induced by exogenous oxidative stress in vitro (Song et al., 2006). They reported a relationship between ascorbic acid and sperm DNA integrity. It is reported that ascorbic acid 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. These reports are in agreement with our findings of vitamin C acid significantly reducing the sperm head abnormality. The protective effect observed in the present study may be attributed to the antioxidant and free radical attack scavenging properties of vitamin C (Miller, 1990; Duthie et al. 1996; Sardas, 2006).

The challenge assay is a cytogenetic approach to measure the repair competence of cells (Oberheitmann et al., 1999). The basic concept of challenge assays is to use the assays to investigate cellular activities after exposing cells to DNA damaging agents. The information is used to evaluate how exposed cells respond to different challenging conditions, what cellular functions are involved in the response, and whether the response can have long-term biological consequences. Different types of cellular responses are useful for these evaluations (Au and Salama, 2006). The deleterious effects of low dose exposure to ionizing radiation arise mainly from the results of initial lesions induced in DNA (Tawn, 1997). Metronidazole is reported to be used as a radiosensitizer in many cases (Pavlov, 1984; Andreev, 1985, Andreev, 1986; Gulverdashvili, 1987; Acharya, 1994; Krause, 2005). Some reports are contradictory (Malomo, 2005). At the same time the phenomenon of adaptation also cannot be overlooked. Adaptation is the phenomenon where pre-exposure to low dose radiation (or some other genotoxic agent) can change
radiosensitivity, alter score of chromosomal aberrations, micronucleus and mutations (Au and Salama, 2006).

Metronidazole sensitized cells exposed to radiation (0.5 Gy) and the radiation (0.5 Gy) sensitized cells treated with metronidazole treatment induced chromosome aberrations in the bone marrow cells of mice (Table–3, Figure–7). But when a comparison was made it was found that as compared to the pre treatment, radiation when given following metronidazole, comparatively higher frequency of chromosome aberrations were observed (Table–3). This finding is in conformity with the earlier studies reporting metronidazole to be a radio sensitizer (Pavlov, 1984; Andreev, 1985; Andreev, 1986; Gulverdashvili, 1987; Acharya, 1994; Krause, 2005). In mammalian cells, reduction of the nitro group of 2-nitroimidazoles (metronidazole is a 5-nitroimidazole) is mediated by NADH, xanthine oxidase, NADPH cytochrome P450 reductase and NADPH cytochrome C reductase. It has been found that DNA helix destabilization and strand breakage induced by reduced nitroimidazoles is related to high content of A + T bases. Metronidazole can be reduced by xanthine oxidase to seven products, but the synthetic amine derivative of metronidazole has been found to be inactive. Electrolytically reduced metronidazole (the specific nature of the molecule is not known) produces extensive DNA damage by loss of helix conformation, strand breakage and possibly by the alteration of bases (Bendesky, 2002).
In bone marrow micronucleus analysis also it was observed that metronidazole treated animals when exposed to radiation (Table–6, Figure–10), there was apparent increase in the frequency of micronucleated cells as compared to the radiation pre treatment groups, although not statistically significant.

In the sperm head abnormality assay (Table–9, Figure–13), both in the short term (24h) as well as long term (21 days) analysis, significantly higher frequency of sperm head abnormality was observed in the combined (metronidazole and radiation) treated groups as compared to the metronidazole treated groups alone. Further there was clearly significant difference between the radiation pretreated groups and the radiation post treated groups, the higher frequency being observed for the radiation post treatment groups (Table–9). The present findings further suggest that the germ cells may be more sensitive to metronidazole and may sensitize the cells to radiation exposure.

5.2 CIPROFLOXACIN

Fluoroquinolones are a group of synthetic antibiotics chemically related to nalidixic acid. These compounds have been developed for the treatment of a variety of microbial infections in both animal and human medicine (Abadia et. al., 1995; Garcia et. al., 1999; McKellar et. al., 1999; Bregante et. al., 1999; Perez et. al., 2002). It is particularly useful in the treatment of respiratory and
urinary tract infections due to multiple pathogens or pathogens resistant to other antibiotics.

Genotoxicity data on ciprofloxacin in mammalian test system are contradictory. It has been reported that ciprofloxacin induces positive genotoxic results in the _Salmonella typhimurium_ TA102 (Gocke, 1991; Mamber _et al._, 1993), SOS response (Gocke, 1991), repair dependent cytotoxicity (Mamber _et al._, 1993) and arrest of DNA synthesis (Sitjes _et al._, 1992). Positive genotoxicity result in the prokaryotic test system with quinolones can be explained as the direct consequence of the therapeutic principle, the inhibition of gyrase, a bacterial enzyme involved in DNA replication. However, gyrase is not found in the eukaryotic cells but gyrase is functionally and structurally related to the eukaryotic topoisomerase-II. Due to this structural relationship, it is important to assess the genotoxic hazard of quinolones using eukaryotic organisms (Herbold _et al._, 2001). Positive results of in vitro cytogenetic studies for ciprofloxacin have also been reported (Theiss _et al._, 1989; Theiss _et al._, 1992).

However, comprehensive program of _in vivo_ genotoxicity tests performed by Herbold _et al._ (2001) reported that ciprofloxacin was neither clastogenic nor did it induce DNA damage. Similar report stating that ciprofloxacin is considered to be safe for therapeutic use was also reported by Bernd _et al._ (2001).
In our study, ciprofloxacin induced different types of chromosomal aberrations in the bone marrow cells. The gaps and breaks were more frequent than other aberrations. Significant dose response correlation could be found at 24 h of the i.p treatment of acute dose. The highest acute dose (50 mg/kg) induced significant increase in the frequency of chromosome aberrations when compared to the untreated control (P<0.001). However, of all the doses, the chronic dose induced significantly (P<0.001) the highest chromosome aberrations when compared to the untreated control (Table-10, Figure-14). This indicates that either the secondary metabolite(s) of ciprofloxacin may induce mutagenic effects or the chemical is slowly degraded and remains persistent for longer durations. The production of chromosome aberrations is a complex cellular process. Structural chromosome aberrations result from: (i) direct DNA breakage, (ii) replication on a damaged DNA template, (iii) inhibition of DNA synthesis, and other mechanisms like topoisomerase II inhibitors (Albertini et. al., 2000). The low and middle dose did not show significant increase in the frequency of chromosomal aberrations. This could be due to the fact that lower doses are more rapidly detoxified thus sufficient quantity of the chemical does not reach the target tissue.

The mechanism of antibacterial action of quinolones is not completely understood; however, it has been proposed that the initial event is the inhibition of DNA synthesis by interference with the nick sealing activity of DNA topoisomerase II (DNA gyrase) and DNA topoisomerase IV. In the presence of
these antibiotics, the enzyme is trapped on the DNA, resulting in the formation of quinolone-enzyme-DNA complexes, and the subsequent release of DNA ends from this complex leads to the generation of “cellular poison” which ultimately leads to cell death (Chen et. al., 1996; Drlica et. al., 1997; Hawkey et. al., 2003).

Results of an in vivo bone marrow chromosomal aberration assay also showed positive results with ciprofloxacin for the doses of 6 and 20mg/kg (Mukherjee et. al., 1993). At high concentrations, some fluoroquinolones have been reported to exhibit genotoxic effects in eukaryotic systems as a result of topoisomerase inhibition (Robinson et. al., 1991; Kohlbrenner et. al., 1992).

Evaluation of micronucleus frequency has been recommended by the regulatory agencies to be conducted as part of product safety assessment (Krishna and Hayashi, 2000). To test somatic mutagens in vivo, bone marrow micronuclei assay has been used extensively (Tice et. al., 1994, Giri et. al., 2002a). Micronuclei are small extra-nuclear bodies that are formed in mitotic cells from chromosome fragments or whole chromosomes lagging behind in anaphase and are not integrated into either of the daughter nuclei (Albertini et. al., 2000). Hence micronuclei test is used as a biomarker to find out the genotoxic potential of a chemical.

In the present study also a significant increase in the frequency of micronucleated PCEs, NCEs and NCs was observed following higher doses of
ciprofloxacin treatment (Table–13). The chronic dose (3x16.7 mg/kg) also induced significant increase (P< 0.01) in the frequency of micronuclei when compared to untreated control. The results of the present study indicate that ciprofloxacin induces the formation of micronuclei in a dose-dependent manner. The formation of micronuclei harboring chromosomal fragments result from: direct DNA breakage; replication on a damaged DNA template; and inhibition of DNA synthesis. Micronuclei harboring whole chromosomes are primarily formed from failure of the mitotic spindle, kinetochore or damage to chromosomal substructures, alterations in cellular physiology and mechanical disruption (Albertini et. al., 2000). The present findings further support the clastogenic potential of ciprofloxacin.

Very few published literature is available on the reproductive effects of ciprofloxacin and especially on its genetic effects. There is also insufficient data about its effects on sperm morphology. Under in vivo conditions, the genotoxicity assessment and in particular, the clastogenic potential of an agent is evaluated using the chromosome aberration assay (Preston et. al.,1987) and analysis of sperm head abnormality can provide further information on the sensitivity of the germ cells to a particular chemical agent as these cells determine the reproductive consequence of an organism.

Sperm abnormality assay is a sensitive and reliable endpoint and is widely used to identify germ cell mutagens (Wyrobek and Bruce, 1978; Giri et.
al., 2002a, 200b; Nahas et. al., 2004). The total sperm count as well as sperm shape abnormalities were observed after 24h and 21 days of exposure of ciprofloxacin.

In the present study, ciprofloxacin induced various forms of sperm head abnormalities, which has been identified and classified as described earlier. As has been observed in case of metronidazole, in the present case also triangular, hook shape, amorphous and banana shape heads were more prevalent than other types. All the doses of ciprofloxacin tested induced significant increase in the frequency of sperm head abnormality both at 24h and 21 day of the exposure (Table–16). The significant increase in the sperm head abnormality observed could be due to interference of ciprofloxacin in the differentiation process of the sperm or interaction with cell membrane components like membrane lipids thus resulting in distortion in the head morphology. Induction of abnormal sperms is presumed to be a result of naturally occurring errors in the differentiation process, or the consequence of an abnormal chromosome complement (Bruce et. al., 1980). The characteristics controlling sperm head shape are carried on the autosomes and sperm abnormality test identifies those agents, which cause small alterations in testicular DNA (Topham, 1980). The highly significant increase in sperm head abnormality observed in the present study following 21 days of the treatment clearly indicates that ciprofloxacin could be a potential germ cell mutagen.
In the combination studies with vitamin C (Table–11, Figure–15), both the acute and chronic doses of ciprofloxacin treated mice showed a decreasing trend in the frequency of chromosomal aberration when pre-treated with vitamin-C but the decrease was not statistically significant. Both the acute and chronic doses of ciprofloxacin treated mice showed decrease in the percentage of micronucleus when pretreated with vitamin C and the decrease was statistically significant with the acute dose (P< 0.001) and chronic dose (P<0.01) (Table–14, Figure–18). Significant reduction in the frequency of micronuclei in the PCEs as well as NCs was also noted for the acute dose of ciprofloxacin (50 mg/kg) when pretreated with vitamin C. In the sperm head abnormality assay in the present study (Table–17), vitamin C pretreatment could not show any significant level of protection, except for the highest acute dose of 50 mg/kg where pretreatment with vitamin C could show some protection at 24h of the treatment. However, at no other tested dose, any significant protection could be observed either at 24h or at 21 day of the treatment. The result found can be compared to the observation in chromosome aberration assay as the trend is similar in both the cases.

Vitamin C is known to be a free radical scavenger. Compounds such as ascorbic acid can readily cross the cell membrane because of their hydrophilic nature, low molecular weight, and presence of specific transporters for vitamin C on the cell membrane (Parry and Clark, 2002; Zhang et. al., 2003), which enables them to manifest their antioxidant action in the cytosol. The general
lack of any appreciable protection by vitamin C indicate that ciprofloxacin induced genotoxicity may not involve reactive oxygen of free radical species. The present findings are in agreement of the earlier reports that the levels of reactive oxygen species generated during the photoactivation of fluoroquinolones are not found to correlate to their genotoxic effects (Umezawa et al., 1997; Martinez et al., 1998).

Photosensitivity is a well known adverse reaction in ciprofloxacin based treatment regimens (Jensen et al., 1987). Patients taking ciprofloxacin are usually advised to protect their skin from direct sunlight. It is reported that more attention should be paid to indoor sources of UV light (Adam and Andrew, 1999). Radiation in general is seen to have an adverse effect with ciprofloxacin. Adverse reactions affecting the skin due to fluoroquinolones have been estimated to occur in 0.5–10% of patients taking the drug, and these reactions tend to be reversible. It is thought that the abnormal skin reaction seen in patients treated with ciprofloxacin is caused by a phototoxic mechanism induced by UV radiation (Adam and Andrew, 1999). Fluoroquinolones are known to induce the formation of singlet oxygen and superoxide anion, which are responsible for the phototoxic effect of the fluoroquinolones (Umezawa et al., 1997). There are reports which indicate that in the presence of ultraviolet (UV) radiation a number of fluoroquinolones exert photochemical toxic and mutagenic effects (Chetelat et al., 1996; Rosen et al., 1996; Martinez et al., 1998; Spratt et al., 1999; Jeffrey et al., 2000; Marrot et al., 2000).
In the present study, ciprofloxacin sensitized animals exposed to radiation (0.5 Gy) and the radiation (0.5 Gy) exposed animals treated with ciprofloxacin induced chromosome aberrations in the bone marrow cells of mice (Table–12). In the 50 mg/kg treated group, significant increase in the frequency of chromosome aberrations was found when these animals were exposed to radiation. Further, in the chronic treatment (3x16.7 mg/kg) group both pre and post irradiation protocol significantly increased the frequency of chromosome aberrations (Table–12).

The deleterious effects of low dose exposure to ionizing radiation arise mainly from the results of initial lesions induced in DNA (Tawn, 1997). The ionizing radiation induces direct DNA damage. These agents induce, at the time of exposure, chromosome type chromosomal aberrations in cells in the G$_0$/G$_1$ phase of the cell cycle and chromatid type chromosomal aberrations in cells in the S/G$_2$ phase. These agents are classified as S-phase-independent clastogens (Albertini et al., 2000).

In the bone marrow micronuclei analysis (Table–15, Figure–19), both the doses tested (50 mg/kg and 3x16.7 mg/kg) induced significantly higher frequency of micronuclei when exposed to radiation either prior to or following ciprofloxacin treatment. Further, significant increase was observed when radiation exposure was given following ciprofloxacin treatment in the chronic treatment group. In the PCEs also similar result was observed. Very few reports
are available on the effect of radiation and ciprofloxacin given in combination. The present findings indicate that ciprofloxacin may sensitize the cells for radiation damage.

In the sperm head analysis (Table–18, Figure–22), it was observed that at 24h of the treatment significantly higher frequency of sperm head abnormality was observed when ciprofloxacin was given with low dose of radiation. Similar result was also observed at 21 day of the treatment. More importantly, it was found that radiation exposure following ciprofloxacin treatment always induces more sperm head abnormality than when ciprofloxacin is given following radiation exposure (Table–18). The present findings provide further evidence that apart from being a potential germ cell mutagen; ciprofloxacin may act in other ways in the short term to bring about distortions in the sperm head morphology and hence may limit their reproductive competence also.

5.3 CHLOROQUINE

Chloroquine is presently the most commonly used anti-malarial drug in different parts of the world. In tissues chloroquine becomes concentrated in lysosomes, particularly in such cells as those of the liver parenchyma. From all these sites they are slowly excreted and metabolized and may be present in tissues for months or even years after discontinuation of therapy (Chatterjee et. al., 1998).
Apart from the quinolone antibiotics, significant controversies exist regarding the genotoxicity of anti-malarial drugs (Chatterjee et. al., 1998; Riccio et. al., 2001; Azas et. al., 2002; Tunca et. al., 2002). The published results of genotoxicity testing of chloroquine are conflicting and contradictory. Chloroquine is reported to induce teratogenic effects in rats (Sharma and Rawat, 1989). Several studies demonstrated mutagenicity of chloroquine in bacterial systems. Report on mutagenicity in Salmonella typhimurium strains including positive responses of chloroquine with TA104 in the absence of metabolic activation, and with strains TA97a and TA100 both with and without metabolic activation has already been published (Chatterjee et. al., 1998). Chloroquine was positive for mutagenicity with tester strain TA1537 (Cortinas de Nava et. al., 1983), whereas mefloquine did not induce mutagenicity at concentrations up to 2.5 mg/ml, either without or with metabolic activation (Schupbach, 1979). Chloroquine was previously reported to be mutagenic with strains TA98 and TA97 (Obaseiki-Ebor and Obasi, 1986; Thomas et. al., 1987). Similar results were reported with other bacterial strains including Escherichia coli strains EE97 and EE102 (Obaseiki-Ebor and Obasi, 1986) and E. coli pol A1/pol A2 strains (Espinosa-Aguirre et al., 1987). In contrast, a negative result in mammalian cell mutagenicity assays for chloroquine has been reported (O'Donovan, 1984). Riccio (2001) reported that although both chloroquine and AQ-13 showed weak bacterial mutagenicity, this mutagenic effect was not
confirmed in either the mouse lymphoma mutagenesis assay or the micronucleus assay.

In the present study, chloroquine induced a variety of chromosomal aberrations in the bone marrow cells such as chromatid and isochromatid type of gaps and breaks and chromatid exchanges (Table-19). The dose response histogram at 24h of i.p treatment (Figure-23) shows a linear increase in the frequency of chromosomal aberrations with increasing acute dose. In a study using sister chromatid exchange and chromosome aberration parameters, Chatterjee et. al. (1998) reported that chloroquine, primaquine and amodiaquine are genotoxic in bone marrow cells of mice. Tunca (2002) also reported that although statistically significant change was not determined in numerical chromosome abnormalities but structural chromosome aberrations increased in a dose-dependent manner by cytogenetic and statistical evaluations following chloroquine treatment. Earlier reports also suggest that chloroquine has the potential to increase chromosome aberrations (Shalumashvili and Sigidin, 1976) and chloroquine phosphate can produce damage to human chromosomes (Neill et. al., 1973).

Evaluation of micronucleus frequency has been recommended by the regulatory agencies to be conducted as part of product safety assessment (Krishna and Hayashi, 2000). To test somatic mutagens in vivo, bone marrow micronucleus assay has been used extensively (Tice et. al., 1994, Giri et. al.,
In the present study chloroquine induced micronuclei in the bone marrow cells of mice (Table–22, Figure–26). Significant increase in the frequency of micronucleated polychromatic erythrocytes (PCEs), NCEs and NCs were found following 3x10 mg/kg of chloroquine treatment as compared to the untreated control group. Significant (P< 0.01) increase in the frequency of micronuclei in the NCs was also noted following 10 and 20 mg/kg of chloroquine treatment. Chloroquine has been reported to induce micronuclei (Tunca, 2002). Micronuclei are small, extranuclear bodies consisting of acentric fragments of chromosomes or entire chromosomes, which lag behind at anaphase of cell division. After telophase, these fragments may not be included in the nuclei of daughter cells and form single or multiple micronuclei in the cytoplasm. The significant increase in the frequency of micronuclei in the bone marrow cells following chloroquine treatment observed in the present study further indicate the mutagenic potential of chloroquine.

The mechanism behind the mutagenic action of chloroquine is not very clear. The therapeutic action of chloroquine is based on its cytostatic and immunodepressive effects, due to interaction between the compound and the nucleic acids of the cells. Chloroquine has been reported to form intercalated complexes with DNA (Cohen and Yielding, 1965; Stemglanz et. al., 1969; Waring, 1970) and acts as an inhibitor of DNA synthesis and repair by inhibiting DNA polymerase (Yielding et. al., 1970; Wichard et. al., 1972; Field et. al., 1978). This marked effect on DNA metabolism makes a cytogenetic analysis of
the action of chloroquine essential (Shalumashvili and Sigidin, 1976). The binding of chloroquine with DNA leads to stabilization of the latter and prevents its denaturation. Intermolecular cross linkages are formed in double stranded DNA molecules and as a result the likelihood of the appearance of chromosomal aberrations is increased (Shalumashvili and Sigidin, 1976).

Sperm abnormality assay is a sensitive and reliable endpoint and is widely used to identify germ cell mutagens (Wyrobek et. al., 1978, Giri et. al., 2002a, 2002b; Nahas et. al., 2004). It was observed in the present study that following 24h of the treatment, all the three doses (i.e. 10, 20 and 3x10 mg/kg of chloroquine) tested, induced significant (P< 0.001) increase in the frequency of abnormal sperms at 24h of the treatment (Table–15, Figure–29). Further, significant increase was found in the 20 mg/kg treated group (P< 0.05) and 3x10 mg/kg treated group (P< 0.01) as compared to the group receiving 10 mg/kg of chloroquine. Germ cells take about three weeks to get differentiated in functional sperms. Therefore, the observed effects after 24h may be of little genetic relevance and indicate that aspirin may interact with cell membrane components thus resulting in the abnormal head morphology at maturation stages. However, significant increase (P< 0.001) in the frequency of abnormal sperms observed in the groups receiving the highest acute dose of 20 mg/kg as well as 3x10 mg/kg of chloroquine treated groups (Table–25) at 21 day of the treatment indicates that chloroquine at higher doses may be considered as a germ cell mutagen.
The role of vitamin C in protecting against oxidative DNA damage is a matter of much controversy (Rivière et. al., 2006). Antioxidant supplements increase the resistance of lymphocytes to oxidative damage (Sardas et. al., 2006). Reports on vitamin C intervention in the mechanism of chloroquine action especially with reference to genotoxicity are scanty. In the present study with vitamin C, it was observed that vitamin C pretreatment apparently reduced the frequency of chromosome aberrations induced by chloroquine. The decrease was found to be significant (P< 0.02) in the groups receiving vitamin C plus chloroquine 3x10 mg/kg. Pretreatment with vitamin C also significantly reduced the frequency of micronuclei induced by chloroquine in the bone marrow cells (Table–23). Moreover, the frequency of micronuclei in the group treated with chloroquine and vitamin C was at par with the control group. The present findings suggest that vitamin C has the potential to protect the cells against chloroquine related genetic damage.

Further, pretreatment with vitamin C drastically reduced the short term (24h) adverse effects of chloroquine so far as the sperm head morphology is concerned (Figure–30). The decrease for both 20 mg/kg and 3x10 mg/kg group was almost between 65 – 70%. Following 21 days of the treatment, significant decrease (P< 0.02) could be observed for the groups receiving 3x10 mg/kg of chloroquine along with vitamin C (Table–26).
Vitamin C is known to be an antioxidant and is able to scavenge free radicals. It has been reported that the distribution of ascorbic acid and its concentration in the organs is subjected to variations depending on introduction of different drugs (Linster, 2007). The protective action of vitamin C is clearly evident in the present study. Song et.al (2006) found a relationship between ascorbic acid and sperm DNA integrity. Reports on prevention of hydrogen peroxide-induced sperm DNA damage by ascorbic acid pretreatment also been published (Donnelly et. al., 1999). It is also reported that oral treatment with vitamin C and E significantly reduced sperm DNA damage (Rolf et. al. 1999; Greco et. al., 2005). These reports are in line with findings of present study using chloroquine and vitamin C.

There are several chemical agents which sensitize the cells to radiation exposure. On the contrary, pretreatment to radiation also sensitizes the cells to many chemical agents. In the literature survey using the internet, no reports could be found on the interaction of chloroquine and radiation so far as interaction of chloroquine with genetic material is concerned.

In the present study, both the doses of chloroquine tested in combination with radiation, induced significantly higher frequency of chromosome aberration as compared to the control group (Table-21). However, no significant difference in the frequency of chromosome aberration could be observed between the groups receiving only chloroquine and those receiving chloroquine along with
radiation. However, in the bone marrow micronucleus analysis (Table–24), significant increase in the frequency of micronuclei was found in the groups receiving 20 mg/kg of chloroquine when exposed to radiation either prior or following chloroquine treatment. The present findings indicate that exposure to low doses of radiation (0.5 Gy) either prior to or following chloroquine, in the dose ranges tested, has little or no synergistic effect in the clastogenic evaluations.

In the sperm abnormality assay also no significant difference could be found in the frequency of abnormal sperms in the groups receiving chloroquine and radiation as compared to the respective groups receiving chloroquine alone (Table–27). However, at 21 days of the treatment, significant increase in the frequency of sperms with abnormal head morphology was observed in the groups receiving the combined dose of chloroquine and radiation as compared to the groups receiving chloroquine alone. Further, it appeared that radiation exposure following chloroquine treatment has more effects as compared to prior exposure to radiation. The present findings suggest that in contrast to bone marrow cells, germ line cells may be more sensitive to chloroquine and radiation.

The mechanism whereby chloroquine produced mutagenic/clastogenic effects is not very clear. However, the findings of the present study are in support of the various reports published earlier indicating positive results on genotoxicity of chloroquine (Shalumashvili and Sigidin, 1976; Shaw, 1970;
Chatterjee et al., 1998; Tunca et al., 2002). Chloroquine is slowly metabolized and excreted from the body and may be present in tissues for months or even years after discontinuation of therapy (Chatterjee et al., 1998). Therefore, in the light of the findings of the present study and those reported earlier, points to the necessity for a special study of the mutagenic effect of chloroquine under clinical conditions.

5.4 ACETAMINOPHEN

Acetaminophen (N-acetyl-p-aminophenol) is the major metabolite of phenacetin (Clissold, 1986). It is an antipyretic and analgesic, frequently administered therapeutically to reduce fever caused by bacterial and viral infections and by clinical trauma such as cancer or stroke. It is often classified as a nonsteroid anti-inflammatory drug (NSAID), but differs from other NSAIDs because it does not reduce inflammation or cause ulceration and bleeding of the stomach mucosa.

At pharmacological doses, acetaminophen is metabolized by sulfation and glucuronidation, and to a lesser extent, by cytochrome CYP2E1 that produces a reactive metabolite, NAPQI, which is detoxified by conjugation with glutathione (GSH) (Dahlin et al., 1984; Mitchell et al., 1973). Acetaminophen-induced hepatotoxicity occurs when GSH reserves are exhausted allowing covalent binding of NAPQI to critical cellular proteins as acetaminophen-
cysteine adducts, ultimately disrupting their cellular function. Many of these covalently bound proteins are within the mitochondria (Qiu and Burlingame, 1998) resulting in reduced respiration (Donnelly et. al. 1994) and increased superoxide production (Jaeschke, 1990). Superoxide either reacts with nitric oxide to produce peroxynitrite, which is responsible for protein nitration (Hinson et. al. 1998), or dismutates to hydrogen peroxide whereby it can oxidize cellular macromolecules. It has been postulated that loss of mitochondrial function and concomitant generation of oxidative stress are central to acetaminophen induced hepatotoxicity (James et al. 2003).

Acetaminophen is mainly conjugated in the liver, and then excreted in the urine. Plasma half-life is 1.5 to 3 hours. There are various incidences of toxicity with acetaminophen. As reported in IARC (1990), a positive association between the use of paracetamol and cancer of the ureter was observed in an Australian case-control study. Paracetamol induced chromatid breaks in peripheral human lymphocytes in vivo. It gave negative results in the micronucleus test in mice in vivo, and did not induce chromosomal aberrations in spermatocytes of mice (IARC, 1990).

It has been reported that the carcinogenicity by oral administration of paracetamol was dependent on the dose of the drug administered and the strain of the test animal (IARC, 1990). Paracetamol was tested for carcinogenicity by oral administration in mice and rats. In one strain of mice, a
significant increase in the incidence of multiple liver carcinomas and adenomas
was observed in animals of each sex at a markedly toxic dose but in another
strain, no increase in the incidence of any tumor was observed at a well-
tolerated dose that was approximately half that in the preceding study. In a
further strain of rats, the incidence of neoplastic liver nodules was increased in
animals of each sex given the higher dose; the combined incidence of bladder
papillomas and carcinomas was significantly greater in high-dose male and in
low-dose female rats. Although treatment increased the incidence of bladder
calculi in treated rats, there was no relationship between the presence of calculi
and of either hyperplasia or tumors in the bladder (IARC, 1990).

There is also report about the Induction of chromosome aberrations in
vivo in bone-marrow cells of albino mice by paracetamol with single and
cumulative dose by the serial dilution of the highest dose of 400mg/kg for i.p
administration and 800mg/kg for oral administration (Severin and Beleuta,
1995). Powell et. al. (2006) have undertaken studies to substantiate the findings
of a gene expression signature for oxidative stress by a sub-toxic dose of
acetaminophen in rat liver using a panel of sensitive biomarkers of oxidative
stress and oxidative DNA damage. Although there are numerous reports exist
on the toxicity of acetaminophen in the hepatic cells but reports indicating
genotoxicity in the somatic cells and germ line cells are very scarce. Further,
there is insufficient data about its effects on sperm morphology.
In the present study, acetaminophen treatment induced different kinds of chromosome aberrations (Table–30), which were apparently dose dependent (Figure–34). It was observed that the frequency of chromosome aberration was significantly higher in the groups receiving the acute dose of 30 mg/kg ($P<0.001$) and the chronic dose of $3 \times 10$ mg/kg ($P<0.02$) of acetaminophen as compared to the untreated control. However, in bone marrow analysis although an apparent increase in the frequency of micronuclei was noted following acetaminophen treatment, but this was not statistically significant as compared to the untreated control group (Table–31). The present findings supports of the investigations made by (Severin and Beleuta, 1995) who indicated that the cellular damage induced by paracetamol leads to expression in chromosome aberrations such as gaps, chromatid breaks etc. The occurrence of comparatively lower frequency of micronucleus as compared to the chromosome aberrations observed in the present study could be due to the fact that the fate of chromosomal fragments is uncertain so far their segregation in micronucleus is concerned. Apart from that, all fragments may not necessarily form visible micronucleus (Savage, 1988).

In the sperm head morphology analysis in the present study, acetaminophen treatment did not induce any significant increase in the frequency of abnormal sperms at 24h of the treatment (Table–34). Membrane lipid peroxidation is thought to bring about morphological changes in sperm head shape in short term. The presence of nitric oxide, which is induced by
acetaminophen (Gardner et al., 1998), is thought to block propagation of lipid peroxidation (O'Donnell et al., 1997). This reason may be attributed to the observed failure of significant increase in the frequency of sperms with abnormal head morphology at 24h of the treatment.

However, at 21 days of the treatment, except for the lowest acute dose of 10 mg/kg of acetaminophen, all the other doses tested including the chronic dose of 3x10 mg/kg induced significantly higher frequency of abnormal sperms as compared to the untreated control group (Table-34). It is probable that the increase in the number of sperm abnormalities is a consequence of genetic damage to spermatogenic cells and could be used as a measure of damage which spermatogenic cells have suffered from any physical or chemical agent (Wyrobek and Bruce, 1975). The significant increase in the frequency of abnormal sperms observed in the present study at 21 days following acetaminophen provides further evidence to the genotoxic potential of acetaminophen.

Vitamin C pretreatment reduced the frequency of chromosome aberration in the bone marrow cells at 24h of the treatment (Table-29). In the combination studies with acetaminophen and vitamin C, no significant difference in the frequency of micronuclei was observed for any of the cell types studied as compared to the untreated control groups (Table-32). Vitamin C pretreatment marginally reduced the frequency of abnormal sperms in numerical terms following 24h of the treatment and at 21 days of the treatment.
also relatively lower frequency of sperms with abnormal head morphology was observed (Table–35). Song et. al. (2006) found a relationship between ascorbic acid and sperm DNA integrity. It is cited that ascorbic acid pretreatment prevented hydrogen peroxide-induced sperm DNA damage (Donnelly et. al., 1999). It is also reported that oral treatment with vitamin C and E significantly reduced sperm DNA damage (Rolf et. al., 1999; Greco et. al., 2005). A deficiency of antioxidants can increase DNA damage caused by free radicals (Ames, 1983; Edge et. al., 1997; Bast et. al., 1998; Moller and Mousseau, 2001). The pre-treatment with vitamin C could decrease the free radical attack on DNA since it functions as a free radical scavenger.

Radiation has been reported to reduce levels of antioxidants that are used for DNA repair because antioxidants are used for removing free radicals that arise owing to radiation (Ben-Amotz et. al., 1998; Ivaniota et. al., 1998; Neyfakh et. al., 1998a, 1998b; Kumerova et. al., 2000). It has been suggested that this reduction in antioxidants may be responsible for increased levels of mutation (Dubrova et. al., 1996; Ellegren et. al., 1997; Kovalchuk et. al., 2000; Moller and Mousseau, 2001).

In the present study, 0.5 Gy of radiation and acetaminophen (Table–30, Figure–30), it was observed that both pre and post exposure to radiation induced significantly higher frequency of chromosome aberration in the bone marrow cells. Although not statistically significant, but the frequency was higher
than the groups receiving acetaminophen alone. In the micronucleus analysis also significant increase in the frequency of bone marrow micronuclei was found in the group receiving the combined treatment of acetaminophen and radiation as compared to those receiving acetaminophen alone (Table–33). The present finding is in support of the reports indicating that acetaminophen sensitized cells are more sensitive to radiation. Using cultures of adult human brain and embryonic rat brain, it was demonstrated by Casper et. al. (2000) that subtoxic doses of acetaminophen increased the sensitivity of the human glioma cells in culture to ionizing radiation.

Radiation is associated with diminished antioxidant defense owing to the use of antioxidants for free-radical scavenging (Ivaniota et. al., 1998; Neyfakh et. al., 1998a; Kumerova et. al., 2000). While these studies provide important information about the links between radiation exposure and antioxidant defense, there is no study available that links the effects of radiation to antioxidant defense and level of efficiency measured in terms of mutation rates and DNA repair at the level of individuals.

In the combination studies with acetaminophen and radiation (Table–36), it was observed that at 24h of the treatment, in the combined treated groups the frequency of abnormal sperms were higher as compared to the untreated control as well as the groups receiving acetaminophen alone. Further, significant difference (P< 0.05) was found between the group receiving 30
mg/kg and the group receiving 30 mg/kg followed by radiation. Nunia et. al. (2007) reported that a significant elevation over normal in lipid peroxidation level was recorded in gamma irradiated mice. Therefore, the present increase in the frequency of abnormal sperms following combined treatment of acetaminophen and radiation at 24h of the treatment could be due to alterations at the cell membrane due to membrane lipid peroxidation stimulated by radiation.

Following 21 days of the treatment, it was found that in all the groups receiving the combined treatment of acetaminophen and radiation, the frequency of abnormal sperms was significantly higher than the untreated control. Statistically significant (P< 0.001) increase in the combined treated group was observed in the group receiving the chronic dose of 3x10 mg/kg when exposed to radiation either prior or following treatment with acetaminophen as compared to the group receiving acetaminophen alone (Table-36).

Mutations are novel genetic variants, and they are supposed to occur at a constant low rate (Lynch et. al. 1999). Individual differences in mutation rates can arise from individual differences in the ability to cope with mutagens and perform DNA repair. These individual differences in levels of antioxidants are related to levels of mutation, as reflected by sperm abnormality. There are reports in support of increase in sperm head abnormality by radiation. Rogers (2005) reported that chemotherapy also can cause sperm abnormalities and birth defects.
The results of the present study indicate that acetaminophen has the potential to cause genetic alterations in the mammalian test system both in the somatic as well as germ cells. Vitamin C pre treatment (in the dose range tested) could only provide limited protection against acetaminophen induced genotoxicity. Further, acetaminophen and radiation may synergistically contribute to the genotoxic potential of acetaminophen.

5.5 NIMESULIDE

Nimesulide is a sulfonanilide non-steroidal anti-inflammatory drug with proven anti-inflammatory, analgesic and antipyretic activity in several widely used animal experimental models. The good tolerability of nimesulide as an alternative drug for use in patients with non-steroidal anti-inflammatory drugs (NSAID) intolerance has been demonstrated in a large number of clinical trials (Senna et al., 1996).

The overview of nimesulide functionality is given by Bennett, who stated that nimesulide has a unique multifactorial mode of action in treating pain, inflammation and fever coupled with good safety and tolerability compared to other NSAIDs (Bennett, 1999). The mechanism of action of NSAIDs is principally due to the inhibition of cyclooxygenase (COX). COX exists in two isozyme forms: COX-1 is a constitutive form of the enzyme, and COX-2, a cytokine-inducible form of the enzyme. Enhanced COX-2 expression was
observed in esophageal, gastric, colorectal, liver, lung, prostate, the head and neck cancers (Zimmermann et al., 1999; Murata et al., 1999). These findings suggest that COX-2 may play an important role in carcinogenesis. It is thought that inhibition of COX-2 activity by NSAIDs as the antineoplastic mechanism of this class of drugs and gastrointestinal complications of using NSAIDs attribute to the inhibition of COX-1.

A number of studies have shown that NSAIDs have an effect of growth inhibition of cancer cells, but the mechanism of the antineoplastic effect of NSAIDs is unknown. An explanation for the antineoplastic properties of NSAIDs was first suggested by Adolphe et al. (1972), and reported that certain NSAIDs were capable of inhibiting the proliferation of cultured HeLa cells by causing cell cycle arrest. It is also possible that mechanisms responsible for the antiproliferative effects of the NSAIDs on cultured cells are multifactorial. Apoptotic cell death is another mechanism that could contribute to reduced cell growth. Additional recent reports have demonstrated that NSAIDs induced apoptosis in different tumor cells (Arber and DuBois, 1999; Wu et al., 2001; Li et al., 2003).

There are reports which indicate the toxicity of nimesulide (Li et al., 2003). The genotoxic potentiality of nimesulide was evaluated in vivo in murine bone marrow cells. In studies done by Khan et al. (2003), human equivalent prophylactic dose of nimesulide was given to animals orally, once daily for seven consecutive days. Metaphase chromosome analysis revealed significant
increase in the frequency of chromosomal aberrations with preference to structural over the numerical ones. It therefore suggested the clastogenic effect of the nimesulide. The molecular mechanism of mutagenesis is yet to be determined (Khan et. al., 2003). Another report stated that the motility of sperm was affected severely after 6h of nimesulide administration that suggested a crucial role of COX-2 towards fertility of mice sperm (Thotakura, 2007).

The reports on the effect of nimesulide in the somatic cells using cytogenetic endpoints are very limited. At the same time, there is scanty literature on its effects in the germ line cells. No report could be found on the effect of nimesulide on the sperm head morphology. In the present study, although the tested doses of 10, 20 and 30 mg/kg, and the chronic dose of 3x10 mg/kg of nimesulide induced apparently higher frequency of chromosome aberration in numerical terms, but these were not statistically significant as compared to the untreated control group (Table–37).

Evaluation of micronucleus frequency has been recommended by the regulatory agencies to be conducted as part of product safety assessment (Krishna and Hayashi, 2000). In the bone marrow micronucleus analysis in the present study, nimesulide in the dose ranges tested failed to induce any significant increase in the frequency of micronuclei at 24h of the treatment (Table–40). The results of the chromosome aberration and micronucleus analysis in the present study indicate that nimesulide has either very weak
potential to induce clastogenic effects in the human equivalent doses. The findings are in contrast to those reported by Khan et. al. (2003). However, the dose and treatment schedule in both the studies may also account for the observed differences.

Sperm abnormality assay is a sensitive and reliable endpoint and is widely used to identify germ cell mutagens (Wyrobek et. al., 1978; Nahas et. al., 2004; Giri et. al., 2002a, 2002b). In the present study, nimesulide treatment induced significant increase in the frequency of sperms with abnormal head morphology (Table–43) at 24h of the treatment. Although increasing frequency was observed with increasing dose, but a significant dose response was not evident. Primary germ cells through equational as well as reductional cell divisions coupled with complex process of differentiation give rise to functional sperms. Therefore, the observed increase in the frequency of abnormal sperms in the present case at 24h of the treatment may be due to reasons other than genetic alterations in the sperm DNA. Thotakura (2007) observed that sperm motility was severely affected after 6h of nimesulide administration. The exact mechanism of the observed abnormality of the sperm head morphology is not known. Several findings support the antioxidant activity of nimesulide (Maffe-Facino et. al., 1993a; 1993b; Maffe-Facino, 1995). Therefore, the possibility of nimesulide directly causing oxidative damage to sperm membrane lipids may also be ruled out.
Significant increase (P<0.01) in the frequency of abnormal sperms as compared to the untreated control at 21 days of the treatment (Table-43), can result from genetic alterations in the spermatogonial cells which subsequently differentiate into sperms. A correlation between germ cell mutations and an increased frequency of sperm shape alterations has been shown by several authors (Wyrobek et. al., 1983). These findings are in contrast to the findings of the bone marrow chromosome aberration as well as micronucleus analysis. These findings indicate that the germ cells may be more sensitive to nimesulide induced genotoxicity due to lowered threshold.

In the combination studies with nimesulide and vitamin C (Table-38, Figure-42), no statistically significant difference in the frequency of chromosome aberrations was found between the combined treated groups and the untreated control. Further, there was no significant difference between the combined treated groups and the respective groups receiving nimesulide alone. Similar results were observed in the bone marrow micronucleus assay following vitamin C and nimesulide treatment (Table-41). In the sperm head abnormality assay, although vitamin C pretreatment brought about reduction in the frequency of abnormal sperms following 24h of the treatment. However, at 21 days of the treatment, no significant difference could be found between the groups receiving nimesulide alone or in combination with vitamin C. Vitamin C is known to have antioxidant properties and is capable of free radical scavenging. The
present findings further indicate that the possible germ cell damage induced by nimesulide may not be mediated by oxidative mechanisms.

Radiation has been proposed to reduce levels of antioxidants that are used for DNA repair because antioxidants are used for removing free radicals that arises owing to radiation (Ben-Amotz et. al., 1998; Ivaniota et. al., 1998; Neyfakh et. al., 1998a, 1998b; Kumerova et. al., 2000). It has been suggested that this reduction in antioxidants may be responsible for increased levels of mutation (Dubrova et. al., 1996; Ellegren et. al., 1997; Kovalchuk et. al., 2000; Moller and Mousseau, 2001).

In the combination studies with nimesulide and radiation (Table–39, Figure–43), it was observed that both the acute dose of 30 mg/kg as well as the chronic dose of 3x10 mg/kg of nimesulide induced significantly (P< 0.001) higher frequency of chromosome aberration as compared to the untreated control when exposed to radiation either preceding or following nimesulide treatment. In micronucleus analysis also (Table–42), significantly higher frequency of micronuclei was found in the combined treated groups as compared to the untreated control.

Radiation induced chromosome aberrations are thought to arise as a consequence of misrejoining of free ends of DNA double strand breaks. In quiescent mammalian cells this process of misrejoining is prevalently taken up by the non-homologous end joining process (Dutta et. al., 2005). Hida et. al.
(2000) suggested that the administration of nimesulide as an adjunct with anticancer agents or irradiation may lead to even greater efficacy with various anticancer agents for the treatment of high-risk patients. The findings of the present study also suggest that nimesulide and radiation may act synergistically in inducing clastogenicity in mammalian cells. This increase in mutations may arise from the direct effects of radiation, indirectly through the effects of a reduction in levels of antioxidants or as a result of their combined effects.

In the combination studies with nimesulide and radiation (Table–45, Figure–49), significantly higher frequency of abnormal sperms were observed only in the groups exposed to radiation following 3x10 mg/kg of nimesulide treatment as compared to the group receiving 3x10 mg/kg alone at 24h of the treatment. However, at 21 days of the treatment significant increase in the frequency of abnormal sperms was found in the groups receiving the combined treatment as compared to the groups receiving nimesulide alone. The observed increased frequency of abnormal sperms in the groups exposed to radiation following nimesulide treatment as compared to the groups receiving radiation exposure prior to the treatment of nimesulide (Table–45) further indicate that nimesulide has the potential to sensitize the cells for radiation induced DNA damage.

Though in some cases nimesulide is indicative of showing toxicity but it is reported to be well tolerated among all age groups of patients and even when
used concomitantly with other drugs (Wober, 1999). However, the Committee for Proprietary Medicinal Products (CPMP, 2004) reported adverse drug reaction reports concerning nimesulide, of which 66 were hepatic reactions, including serious reactions (e.g. hepatitis, hepatic failure, including two cases which required liver transplantation).

The results of the present study provide further evidence in support of nimesulide not causing (or very weak) genetic alterations in the somatic cells of mammalian test system with the human equivalent very short term chronic as well as acute doses. The effect of radiation was highly pronounced in the somatic cell for both the acute and chronic doses and the germ line cells also showed an increase in aberration with radiation. Vitamin C intervention plays a vital role in reducing the risk of mutagenicity in both the somatic and germ cells.

5.6 ASPIRIN

Aspirin or acetylsalicylic acid (acetosal) is a drug in the family of salicylates often used as an analgesic, antipyretic and anti-inflammatory agent. It has also an anticoagulant ("blood-thinning") effect and is used in long-term low-doses to prevent heart attacks. There are several reports on the various adverse effects of aspirin but reports on the genotoxicity in the somatic line cells and germ line cells is extremely rare. Aspirin is rapidly hydrolyzed in the body to
salicylate, which is eliminated slowly and can therefore accumulate in the circulation following repeated aspirin treatment (Rowland et. al., 1972).

There are reports on the chemoprotective role of aspirin in the colorectal cancer which might extend to other gastrointestinal cancers such as esophagus and stomach (Antonio, 2003). Similar other reports have also been published (Thun et. al., 1993; Giovannucci et. al., 1995; Li et. al., 2000). Further, it is reported that the aspirin, phenacetin, caffeine mixture failed to induce micronuclei above the background level (Dunn et. al., 1987). The genotoxicity reports on aspirin using cytogenetic endpoints are very scanty. From our literature survey no reports could be found on the genotoxicity study in germ line cells following aspirin treatment.

In the present study, aspirin in the dose ranges tested did not induce any significant increase in the frequency of chromosome aberration in the bone marrow cells (Table–46). Further no significant difference was observed between any of the treated groups. Micronuclei are small extra-nuclear bodies that are formed in mitotic cells from chromosome fragments or whole chromosomes lagging behind in anaphase and are not integrated into either of the daughter nuclei (Albertini et. al., 2000). Hence micronucleus test is used as a biomarker to find out the level of genotoxicity of a chemical. In the micronucleus analysis (Table–49, Figure–53), aspirin did not induce any significant increase in the frequency of micronuclei in any of the cell types.
studied as compared to the untreated control. The present findings did not indicate any positive genotoxic potential of aspirin. Niikawa et al. (2001) reported that aspirin has a suppressive effect on chromosome aberrations induced by mitomycin C in mice. In another study, it has been reported that the mixture of aspirin, phenacetin and caffeine failed to induce micronuclei above the background level (Dunn et al., 1987).

It is probable that the increase in the frequency of sperm abnormalities is a consequence of genetic damage to spermatogenic cells and that this assay could be used as a measure of damage which spermatogenic cells have suffered from any physical or chemical agent (Wyrobek and Bruce, 1975; Giri et al., 2002a, 2002b; Nahas et al., 2004). Sperm abnormality assay revealed that following 24h of the treatment (Table–52, Figure–56), the highest acute dose of 30 mg/kg as well as the chronic dose of 3x10 mg/kg of aspirin induced significant (P< 0.001 and 0.02 respectively) increase in the frequency of abnormal sperms. Since sperms do not undergo any cell division and genetic changes can take place when cells are exposed to mutagens only at the dividing stage, the present increase at 24h may be due to causes other than genetic alterations. However, the significant increase in the frequency of abnormal sperms observed at 21 days of the treatment (Table–52) strongly indicate genetic alterations in the sperm DNA which might have taken place during the development of the spermatogonial cells. It has been suggested that abnormal sperms may arise from the interference of the test substance with the
genetically controlled differentiation of the sperm cells (Rai and Vijaylaxmi, 2001).

The result of the sperm head abnormality test is in contrast to those observed in the somatic cells such as the bone marrow cells. Toxicology studies, in themselves, are quite complex with sources of variability resulting from the dose and delivery of the chemical under study, the choice of animal species, and the differences in biological and pathological responses of various tissues (Boorman et. al., 2002). The present findings suggest differential sensitivity of somatic and germ cells to aspirin.

Vitamin C pretreatment resulted in apparent decrease in the frequency of chromosome aberrations in numerical terms in the combined treated groups (Table–47). Similar results were also obtained in the bone marrow micronucleus assay (Table–50). Aspirin failed to induce statistically significant increase in the frequency of chromosome aberrations or micronucleus in the bone marrow cells. But, further decrease in numerical terms in these parameters followed by vitamin C pretreatment was an expected outcome. Vitamin C, which is a known antioxidant has been reported to protect against chemical mutagens (Giri et. al., 1998).

In the combination studies with aspirin and vitamin C (Table–53, Figure–57), no significant difference in the frequency of abnormal sperms could be found between the groups receiving the combined treatment and the groups receiving aspirin alone either at 24h or 21 days of aspirin treatment clearly
indicating a tissue specific variation in the response in somatic and germinal cells.

In the combination studies with aspirin and radiation (Table-48, Figure-52), it was observed that exposure to radiation either prior to or following treatment of aspirin (both 30 and 3x10 mg/kg) induced significant increase in the frequency of chromosome aberration in the bone marrow cells. Significant increase (P< 0.001) was found in the groups receiving radiation plus 30 mg/kg of aspirin, and the groups receiving aspirin (30 mg/kg) plus radiation (P< 0.001) as compared to the group receiving 30 mg/kg of aspirin alone. In the bone marrow micronucleus assay also similar results were noted (Table-51). Further, it was observed that the frequency of chromosome aberrations were apparently higher in the groups receiving the radiation exposure following aspirin treatment as compared to the groups receiving the radiation dose before aspirin treatment. It has been reported that aspirin-pretreated HeLa TG cells exhibit enhanced sensitivity to radiation and has additive role for amplifying the radiotherapeutic effect (Kim et. al., 2003). The results of the present study also indicate an additive effect between aspirin and radiation in inducing clastogenic effects in bone marrow cells of mice.

In the studies with sperm head abnormality, the combined treatment of aspirin and radiation (Table-54, Figure-58) induced significantly higher frequency of abnormal sperms at 24h of the treatment. Similar results were also
noted at 21 days of the treatment. It has been noted that the frequency of abnormal cells is generally higher in the groups receiving radiation exposure following aspirin treatment as compared to the groups receiving prior to aspirin. Radiation is reported to cause sperm abnormalities (Rogers, 2005). The present findings further support that aspirin and radiation act in a synergistic manner.

The results of the present study indicate that aspirin may be considered to be a safe drug for somatic cells so far as its mutagenic potential is concerned. However, further studies are required on the mutagenic potential of aspirin in germ line cells. Vitamin C may provide limited protection against aspirin genotoxicity in the germ cells. Further, apart from earlier reports, the present study provides further evidence in support of a synergistic action of aspirin and radiation in inducing genotoxicity in mammalian cells.