5.1 Metronidazole

Genotoxicity studies have been commonly used as sensitive biological indicator in the mutagenic bioassays of a drug. Since patients or hospital workers in the radiological diagnosis section undergoing metronidazole medications may get exposed to low dose of radiation, the interaction between drug and physical mutagen in inducing genotoxicity is important. Thus in the present study these parameters were investigated.

Metronidazole at single and fractionated dose induced a variety of chromosomal aberrations in the bone marrow cells (Table-1, 2; Figure-3). The gaps and breaks were more frequent than other aberrations. Significant dose response correlation could be found at 24hr of the i.p. treatment of metronidazole (Table-1, 2, Figure-8). It has been reported that chemicals in general produce the highest frequency of aberrations in rodents 24hr after single exposure, which roughly coincides with the normal length (22-24 h) of the cell cycle (Schmid, 1973). 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) and Nahas and Ashmawy (2004). 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). 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) leading to oxidative stress.

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, 2002b). 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 were observed following metronidazole treatment (Table−11, Figure−4). Significant correlation between the single as well as fractionated dose and the frequency of micronucleated PCEs at 24hr of the i.p treatments was observed. 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 morphology has been repeatedly used as indicator of toxicity and mutagenicity in mammals (Hemavathi and Rahiman, 1993; Khan and Sinha,
1996; Bustos-Obregón and González-Hormázabal, 2003; Joshi, et al., 2003). The sperm head abnormality assay is a sensitive and reliable parameter and is widely used to identify germ cell mutagens (Wyrobek and Bruce, 1978; Giri, et al., 2002a, 2002b; Nahas and Ashwamy, 2004).

In the present study, single and fractionated dose of metronidazole induced significant increase in the frequency of sperm head abnormality after 35 days of treatment (Table-16; Figure-5). Germ cells take about 35 days to get differentiated into functional sperm. This is the minimum time required to visualize the DNA damage which might have taken place during the development of the spermatogonial cells. 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). 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 24hr of the treatment (Table-16; Figure-18) may indicate interaction of metronidazole with cell membrane components causing distortions in a non-genetic manner.

The results of Chromosomal aberration assay, micronucleus assay and sperm head abnormality showed the genotoxic potential of metronidazole which was confirmed by comet assay. Comet assay is highly sensitive and reliable technique for estimating DNA damage. In the comet assay studies, the metronidazole treatment leads to an increase in tail length, which is at per with other in vitro data on metronidazole, induced DNA damage (Gisell, et al., 2002; Re, et al., 1997; Menéndez, et al., 2001). These results could indicate that metronidazole are direct genotoxic compounds and have clastogenic activity in vitro and in vivo. (Mudry, et al., 1995).
Liver is the largest organ and metabolic center of the body. Drugs and other substances get metabolized in the liver, thus making it susceptible for toxicity from these compounds. Certain drugs when taken in overdose or within therapeutic range may cause tissue damage. Thus oxidative stress related indices in liver tissues were carried out to ensure the probable role of ROS on antioxidant status. Metronidazole lead to increase in TBARS levels in mice thus inducing lipid peroxidation. The results is similar with other studies were metronidazole was observed to cause increase in lipid peroxidation in mice testis (Ligha, et al., 2011). Metronidazole was also found to lead to depletion in GSH which is as per with studies were hypoxic incubation of cells with metronidazole (nitroimidazoles) results in GSH depletion (Varnes, et al., 1980). Similarly decrease in SOD levels were also observed in metronidazole. The reason might be the scavenging of the reactive oxygen species by the GSH and SOD.

Metronidazole sensitized cells exposed to radiation as well as X-ray (0.5 Gy) induced chromosome aberrations in the bone marrow cells of mice (Table 3-10; Figure 9, 10, 11 and 12). This finding is in conformity with the earlier studies reporting metronidazole to be a radio sensitizer (Pavlov, et al., 1984; Andreev, 1985; Andreev, et al., 1986; Gulverdashvili, et al., 1987; Acharya, 1994; Krause, et al., 2005). Increase in the frequency of micro nucleated PCEs were found following both single and fractionated doses of metronidazole and gamma radiation as well as X-ray in combination as compared to the untreated control (Table 12, 13, 14 and 15; Figure 14, 15, 16 and 17. PCE counts in peripheral blood or bone marrow are the most popular and convenient method of monitoring erythropoiesis. Decreases in the proportion of immature PCE to mature or NCE gives us a view of mutagen-induced cytotoxicity (Suzuki, et al., 1989).

In the sperm head abnormality assay (Table 16, Figure 18), both in the short term (24 hr) as well as long term (35 days) analysis, significantly higher frequency of sperm head abnormality was observed in the combined (metronidazole with radiation and X-ray) groups as compared to only
metronidazole exposed groups. Further a decrease in sperm count in the exposed group was also observed. This observation is in accordance with other reports of cytotoxic effects of metronidazole on total sperm count and sperm head abnormality assay (Karbalay-Doust and Noorafshan, 2011). Low dose of gamma radiation lead to significant increase in SHA in mice which is in accordance with other studies (Woon, et al., 2011). The present findings further suggest that the germ cells are very sensitive to metronidazole which may sensitize the cells to radiation exposure.

The percentage contents of DNA in the "comet tail" increased with gamma radiation alone, similar results were reported with other studies (Hossein, et al., 2007; Ramachandran, et al., 2011). The genotoxic activity observed under these experimental conditions is evidenced by the drug’s ability to induce primary DNA damage. This is in agreement with the evidences reported by Re et al., (1997) who found positive results with alkaline gel electrophoresis in metronidazole under in vitro aerobic conditions. These authors proposed that the production of DNA single strand breaks and alkali labile sites in lymphocytes are the result of the so called ‘futile’ cycle, where ROS generation is considered responsible for the production of oxidative DNA lesions (Mason and Holtzman 1975; Lafleur, et al., 1986).This might be the possible reason for genotoxic damage by nitroimidazoles in vivo.

The effect of metronidazole in inducing oxidative stress by inducing ROS was also carried out. This is in accordance with other reports on increase in TBARS levels (Bhatia, 2004; Srinivasan et al., 2006; Yasir, et al., 2010; Sehgal, et al., 2012). This may be due to free radical assault on the fatty acid component of membrane lipids (Prasad, et al., 2005). SOD and GSH decreased significantly in the gamma irradiated group (Yang Liu, 2007). The decrease may be due to radiation induced production of free radicals which in turn can impair the antioxidant defense mechanism leading to an increased membrane lipid peroxidation. Glutathione is the most important intracellular defense against damage by reactive oxygen species. It is a tripeptide (glutamyl-cysteinyl-glycine).
The cysteine provides an exposed free sulphydryl group (SH) that is very reactive, providing an abundant target for radical attack. Reaction with radicals oxidizes glutathione, but the reduced form is regenerated in a redox cycle involving glutathione reductase and the electron acceptor NADPH. Several groups have shown that most reactive electrophiles, including many hepatotoxic drug metabolites, produce effects similar, if not identical, to reactive oxygen species (Nelson, 1995; Rushmore et al., 2002).

The results revealed increased levels of TBARS in metronidazole alone and X-ray treated mice liver tissue. This may be due to the attack of free radicals on the fatty acid component of membrane lipids (Prasad, et al., 2005). The antioxidant enzymes like GSH and SOD plays important role in ROS scavenging. (Turner, et al., 2002). In this study, we have observed a significant decrease in the activities of SOD and GSH in metronidazole treated mice liver tissue. The decrease might be due to radiation induced production of free radicals which in turn can impair the antioxidant defense mechanism, leading to an increased membrane lipid peroxidation (Bhatia, 2004; Sehgal, et al, 2012;). The decreased GSH levels may be due to their utilization by the enhanced production of ROS (Suryanarayana et al., 2007; Balamurugan et al., 2009). All organisms have their own cellular defense system, composed of both enzymatic as well as non-enzymatic components. Since GSH is utilized for detoxification of free radicals, increased sensitivity to oxidative stress also occurs when cells are depleted of GSH (Chen, et al., 1997, Sehgal, et al., 2012; Ginpreet, et al., 2012).

In the antioxidant supplementation study, protective role of vitamin C (L-ascorbic acid) in metronidazole treated mice alone and in combination with low dose gamma radiation (0.5Gy) and x-ray (0.5 Gy) was investigated. CA, MN, comet assay was used to study protections against genotoxicity, SHA and total sperm count was carried out to assess protection against the reproductive toxicity and restoration of endogenous protectors biochemically taking total GSH, SOD was carried out. Lipid peroxidation assay was used to evaluate the role of the antioxidants against ROS induced membrane destabilization. Our
observation indicates that Vitamin C supplemented animals prior to treatment with metronidazole reduced the frequency of chromosome aberrations in the bone marrow cells (Table-1, 2; Figure-8). Similar level of significant protection could also be observed in the frequency of micronuclei in the bone marrow cells (Table-11, Figure-13). In the sperm head abnormality assay also vitamin C conferred significantly protection at 35 days of the treatment (Table-16, Figure-18). It also showed protective role against DNA damage (Table22, Figure-24). Vitamin C pretreatment increased the TBARS level and replenishing GSH and SOD levels (Table-28,31 and 32; Figure-27,30 and 33). Ascorbic acid, a major water-soluble antioxidant, has recently been reported to protect sperm DNA from the damage induced by exogenous oxidative stress \textit{in vitro} (Song, \textit{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, \textit{et al.}, 1999). Rolf, \textit{et al.} (1999) and Greco, \textit{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 (Duthie, \textit{et al.} 1996; Sardas, 2006).

Similarly, Curcumin 8mg/kg bw pretreatment lead to decrease in incidences of chromosomal aberration, micronucleus in PCEs as well as sperm head abnormality. Further it lead to decrease in TBARS level as well as elevation in levels of GSH and SOD. Curcumin pretreated group with metronidazole alone and in combination with gamma radiationas well as X-ray resulted in decreased lipid peroxidation (TBARS). This may be due to the antioxidant scavenging action of curcumin. The presence of pi-conjugation in curcumin makes it more hydrophobic. Thus curcumin get localized in the lipid bilayer membrane (Srinivasan, \textit{et al.}, 2006). Curcumin, being soluble in lipid, reacts with the lipid peroxyl radicals and leads to chain termination. Curcumin is
known to inhibit radiation induced lipid peroxidation in rat liver microsomes (Khopde, 2000). Reports have already shown that curcumin significantly increase the synthesis of antioxidant enzymes such as SOD, catalase in rat liver (Reddy, 1994). Pretreatment with curcumin prior to irradiation protect GSH depletion resulting from the radiation effect. The elevation in GSH content in the metronidazole and/or radiation exposed cells after curcumin treatment suggests that curcumin stimulates the gamma-glutamyl cysteinyl synthase, the rate limiting enzyme involved in the glutathione biosynthesis (Dickinson, et al., 2003). Curcumin has been found to show promising increase in superoxide dismutase (SOD) activity, with comparable free radical scavenging ability and an improved antioxidant efficacy (Barik, et al., 2005; Girish, 2012; Sehgal, et al., 2012). However, when the total antioxidative profile of both Vitamin C and curcumin was compared in the current study, Vitamin C mostly shows more potency as protector over curcumin.

5.2 Artesunate

Artesunate is an antimalarial drug, especially used against chloroquine resistant malaria. Artesunate is made of endoperoxide bond, which is the functional group responsible for the antimalarial activity (Ipcia. Larimal Drug Leaflet). Our study indicates the genotoxic effect of artesunate in the tested dose range, which is dose dependant. Being a newly marketed drug there are not many reports against artesunate indicted clastogenicity (Aquino, et al., 2011), taking CA as endpoint. In the studies by Berdelle, et al., 2011 artesunate was found to be a powerful inducer of oxidative DNA damage in vitro, were it was able to induce 8-oxo-guanine and 1, N 6-ethenoadenine, which are main oxidative DNA base adducts. 8-oxo-guanine is a mispairing lesion and is considered to be responsible for the induction of point mutations following ROS exposure (Klaunig, 2004, Lindahl, 2000 and Nyaga, 2007). This indicates the possible role of artesunate as mutagenic agent, which should be considered as side effect of its use as an antimalaria drug. N 6-ethenoadenine is likely a replication-blocking lesion (Pourquier, et al., 1998; Basu, et al., 1993) which when
not repaired in time, may cause stalled DNA replication that ends up in double
strand breaks (DSB) and activation of the DNA damage response (DDR). Mota,
et al., 2011 also observed a significant increase in the level of DNA damage in
lymphocytes exposed artesunate. These studies possibly indicated that single-
strand breaks induced by artesunate do not undergo repair and are converted
into double-strand breaks. This damage leads to structural chromosomal
aberrations (Natarajan, 2002) and the formation of the observed nucleoplasmic
bridges (NPBs). Artesunate also induces DNA breakage in a dose-dependent
pattern in the studies by Li, et al., 2008. The genotoxic effect was also confirmed
by measuring the level of gamma-H2AX (one of several genes coding histone
H2A), which is considered to be an indicator of DNA double-strand breaks
(DSB). Some contradictory reports about the antioxidant mediator properties of
artesunate have also been reported. Findings of Ho, et al., 2012 showed that
artesunate helped in the treatment of asthma by the amelioration of oxidative
damage in allergic airways, and thus may act by suppressing pro-oxidants and
restoring the activities and expression of antioxidants via activation of Nrf2.
However in our study we observed the pro-oxidant properties of Artesunate
either singly or when given in combination with radiation.

The present study also revealed a significant increase in the frequency of
micronucleated PCEs following artesunate treatment (Table–43, Figure–41).
Incidence of lower frequency of micronuclei than chromosome aberrations could
be due to the fact that the fate of chromosomal fragments is uncertain as far as
their segregation in micronuclei is concerned as all fragments do not necessarily
form visible micronucleus (Savage, 1988). The results of the present study are in
agreement with the studies reported earlier where artesunate showed weak
genotoxic effects at low doses and clastogenic effects at high doses (Aquino, et
al., 2011). Similarly in vitro studies of Mota, et al., 2011 showed significant
increase in the frequency of MN at all tested concentrations which were dose
dependent.
The results obtained from sperm head abnormality assay showed significant reduction in total sperm count with artesunate treatment (Table-48; Figure-46). The incidence of increased abnormal sperm head morphology after 24 hr of exposure may not be attributed to genotoxic effect rather it may be the effect of drug on cell membrane components at maturation stage. However, the germ cells take about 35 days to get differentiated into functional sperm. This is the minimum time required to visualize the DNA damage which might have taken place during the development of the spermatogonial cells. Increase of abnormal sperm head abnormality after 35 days of treatment indicate genetic alterations in the sperm DNA which might have taken place during the spermatogenesis. The significant increase in the sperm head abnormality (Table-48) induced by artesunate indicates its probable potential as a germ cell mutagen. Artesunate has also been shown to cause a decrease in sperm motility in guinea pigs (Obianime, et al., 2009). The effects of artesunate on female reproductive system have also been reported. It was shown to reduce significantly serum progesterone concentration and degenerate the decidual cells and fetus of treated pregnant rats (Lou, et al., 2001). Artesunate has also been reported to cause significant embryo-fetal toxicity causing embryo deaths and malformations in rat (Rath, et al., 2010; Clark, 2009). Further toxicity reports on the testis of rat were artesunate suppress spermatogenesis (Jewo, et al., 2008) has also been reported. Studies by Izunya, et al., 2010 also showed that artesunate could cause some degeneration in the rat testes. This was shown by the sloughing of the germinal epithelium from the basement membrane and reduction in the population of the germ cells. These changes were apparently dose dependent.

The underlying process determining the role of artesunate in destroying Plasmodium vector is a free radical reaction (Olliaro, et al., 2001) which alkylates the parasite’s membranes. The significant reduction in the total sperm count of mice treated with artesunate could be due to the free radical generating capacity of the drug. The probable reason for the occurrence of abnormal sperm morphology in the treated mice could be the radical induced oxidative stress.
Mutagenicity as indicated in our mutation factor and mutation index (Table-53; Figure-51). It was observed that the treatment also increased mutagenicity (or mutation indices) by at least 3.0 fold and the mutation index to 1.8. Thus sperm head abnormality was found to be proportional to mutation index (Ekaluo, et al., 2009).

Alkaline single cell gel electrophoresis was carried out as described by Singh et al. 1988. According to the assay, number of alkali labile sites and single-strand breaks is proportional to a number of DNA fragments and to distance DNA migrated from the nucleus after alkali electrophoresis of agarose-immobilized single cells. The comet assay results revealed the significant increase in tail length following artesunate treatment which is at per the other in vitro studies (Berdelle, et al., 2011; Li, et al., 2008 and Mota, et al., 2011) where DNA damage was observed with artesunate.

Most of the drugs are metabolized in liver leading to the generation of free radicals. Our biochemical estimation in hepatic tissue indicates increased levels of TBARS in artesunate treated mice. This may be due to the attack of free radicals on the fatty acid component of membrane lipids (Prasad, et al., 2005). Generally, artesunate exerts its anti-malarial activity by the generation of reactive oxygen species (ROS) from its endoperoxide bond (Maggs, et al., 1988). This may be the leading cause of lipid peroxidation (Robert, et al., 2001). The accumulation of lipid peroxides is toxic to the membrane structure, leading to a change in permeability and to disintegration of cellular organelles (Muller and Ohnesorge, 1982). In this study, artesunate may have also acted indirectly through generation of high levels of ROS or directly as toxin to the cells of the liver causing membrane destabilization. Necrotic effect of artesunate on the liver was demonstrated by Olliaro, et al., 2001, was a dose dependent increase in malondialdehyde level of the liver was observed. Depressed levels of GSH and SOD were observed following artesunate treatment in our study. The depressed level of GSH and SOD on the liver is possibly due to ROS generation. Another possible reason for lowering of GSH and SOD following artesunate treatment is
possibly their utilization in scavenging the ROS generated by other mechanism. The other studies have revealed that artesunate is a substrate of the glutathione detoxification system (Efferth, et al., 2005).

The results of antioxidant supplementation studies reveals that both vitamin C and curcumin reduce the genotoxic effects of artesunate by decreasing the incidences of CA, frequency of micronucleus, sperm head abnormality and DNA damage. Moreover the antioxidant supplementation leads to elevation in GSH and SOD as well as decrease in TBARS level. Studies by Reddy, et al., 2004 showed that curcumin administration to mice infected with malaria parasite *Plasmodium berghei* reduces blood parasitemia by 80%-90% and enhances the survival significantly.

Except for patients, medical employees such as nurses, pharmacy personnel and staff working in radiological units might be exposed to low doses of radiation while on artesunate medication. Novel strategies are being developed to overcome an irradiation resistant phenotype that may help to increase therapeutic efficacy in cancer cell. Recent studies aimed to elucidate radiation sensitizing properties of artesunate have come forward were artesunate exhibited potent radiosensitivity against human A549 cells *in vitro*, probably via NO signal transduction pathway to induce cell cycle arrest at G2/M phase (Zhao, et al., 2012). Similarly artesunate selectively down regulates survivin (a member of IAP family of antiapoptotic protein) that contributes to a radio-sensitization of glioma cells by an increased induction of apoptosis, cell cycle arrest, and hampered DNA damage responses (Reichert, et. al., 2012). Thus it was interesting to find out what would be the possible effect of irradiation (gamma radiation and X-ray 0.5 Gy) to the cells prior exposed to artesunate *in vivo*. In the drug-mutagen interaction studies, artesunate in combination with low dose of irradiation (both gamma radiation and X-ray) showed increase in the incidences of CA. There was also increase in frequency of micronucleus and abnormal sperm population. The results of comet assay revealed DNA damage with significant increase in tail length in the artesunate
group exposed to gamma radiation and X-ray. Further results obtained from the hepatic biochemical estimations revealed increased TBARS level indicating oxidative stress induced lipid peroxidation. Decrease GSH and SOD levels indicated targeting enhanced radical scavenging. Efferth, et al., 2005 showed that artesunate is a substrate of the glutathione detoxification system. Other report of Mukanganyama, et al., 2001a and 2002b, have also shown that the activity of glutathione S-transferases was inhibited by artemisinin, from which artesunate is derived and that glutathione S-transferases may contribute to the metabolism of artemisinin. The authors also proposed a model in which artemisinin reacts with GSH resulting in oxidized glutathione; which is then converted to reduced glutathione via glutathione reductase; and the latter reaction may then result in the depletion of NADPH by glutathione reductase.

Pretreatment of the artesunate treated group exposed to gamma radiation and X-ray with vitamin C and curcumin resulted in protection from genotoxic damage. The antioxidant supplementation leads to decreased incidences of CA, frequency of micronucleus, sperm head abnormality, DNA damage, and elevation in GSH and SOD and decrease in TBARS level.

The results thus show the efficacy of the antioxidants vitamin C and curcumin in ablating the genotoxic effects of artesunate in combination with gamma radiation and X-ray.