DISCUSSION

The present study was undertaken to see the involvement of different factors in pathogenesis of colon cancer and to study the possible role of selenium in modulating the changes induced by DMH. Keeping in view the above aim, studies were performed to achieve the following objectives: estimation of activities of enzymes involved in the antioxidant defense system; alkaline phosphatase; status of a lipid peroxidation; apoptosis study; micronucleus assay; total and lipid bound sialic acid estimations; colon selenium estimations; biophysical indices; histopathological and ultrastructural studies during selenium treatment to DMH treated rats.

Body weights

In the present study, we observed a progressive increase in the body weights of normal animals at the end of two durations of 10 and 20 weeks study in comparison to their initial body weights. Though, the net body weight gain of the DMH treated animals was markedly less when compared to the normal control animals at the end of the study. This could be due to increased peroxidation of lipids as a consequence of oxidative stress in the animals following DMH treatment. The Steinhoff et al., (1990) have also reported a decrease in the body weights of the DMH treated animals. Li and Li, (2005) have shown the body weights of the rats treated with DMH were significantly decreased compared to the normal controls. Moreover, earlier report from our lab has also shown a significant decrease in body weights of the animals subjected to DMH treatment (Vijata Dani et al., 2008). The appearance of this effect in animals exposed by other routes suggests that appetite may be decreased. The decrease in body weights could also be attributed to alterations in fat metabolism as a consequence of increased

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gluconeogenesis in order to meet increased energy requirement for hyperplastic cells. Alternatively, decreases in body weight gain may be secondary to an underlying disease like cancer. Since, we have not noticed any appreciable change in the diet consumption, the decreased body weight following DMH treatment may be attributed to overall increased degeneration of lipids and proteins. The increase in oxidative stress following DMH administration may be the reason for loss of body weight.

Further, the supplementation of selenium along with DMH significantly increased the body weights of the animals when compared to the DMH treated animals at the end of both 10 and 20 weeks of study. In this context, Maryce et al., (1981) have reported that selenium supplementation to DMH treated rats for a period of 21 weeks increased the body weights of the animals. However, the body weights of the animals with selenium alone was not significantly different from the normal controls. The improvement in body weights following selenium supplementation may be attributed to the antioxidant properties of selenium.

**Aberrant crypt foci (ACFs) and Tumor analyses**

Aberrant crypt foci (ACFs) are preneoplastic lesions and that those with a crypt multiplicity of more than 4 continue to grow into tumors (Bird, 1995). ACFs have been used as an index of preneoplastic lesions in the colons (Takahashi et al., 1993). It may offer the opportunity to observe the very earliest molecular alterations on the multi-step pathway to colorectal cancer (CRC). To date, hundreds of publications on this basic biology in animal models have appeared, and the number is growing rapidly. In the present study, DMH treatment for 10 weeks did not reveal any tumours but caused the formation of ACFs while upon selenium supplementations, were appreciably reduced. A similar inhibitory effect
of sodium selenite on ACF formation has also been reported earlier (Davis and Uthus, 2002). Hu et al., (2008) have reported that selenium-enriched milk protein significantly increased plasma selenium as well as increased the acute apoptotic response to azoxymethane, reduced cell proliferation and frequency of K-ras mutations in ACF. The inhibitory action of selenium could be explained by its putative antioxidant, antiproliferative activity and also its important role in apoptosis.

1,2-dimethylhydrazine are chemical carcinogens which, can produce colorectal tumor lesions in almost 100% of treated animals (Shirai et al., 1983; Veceric and Cerar, 2004). However, various strains of rats differ in susceptibility to these carcinogens (Veceric and Cerar, 2004; Kobaek-Larsen et al., 2002). In the present study, selenium treatment to DMH treated rats for 20 weeks caused a reduction in the tumor incidence, tumor multiplicity, with a concomitant reduction in average tumor size thereby strongly suggesting the potential role of selenium in inhibiting/ slwoing tumorogenesis induced by DMH in the rat colon. In an earlier study Reddy et al., (1987) have reported a reduction in the tumor incidence, tumor multiplicity and tumor size following selenium supplementation to DMH treated animals. Moreover, absence of tumor incidence in selenium treated rats suggests that selenium at this dose level caused no disruption of normal cellular homeostasis and hence was non-toxic. The inhibition of DMH induced colon carcinogenesis by selenium maybe due to attenuation of metabolic activation and detoxification of DMH and/or to its ability to induce the selenoenzyme glutathione peroxidase activity.
Selenium levels in the colon tissue

Selenium is an essential trace element in human and animal nutrition, and it plays several important roles in the form of selenoproteins, including the families of glutathione peroxidase, deiodinases and thioredoxin reductases (Driscoll and Copeland, 2003). Besides nutritional roles, selenium is thought to be associated with cancer prevention, judging by the results of epidemiological studies (Schrazuer, 2000). Cindy et al., (2002) have reported the selenium levels were decreased in the serum and colon of the selenium deficient group when compared to the selenium supplemented group of DMH treated animals. In the present study selenium levels decreased in colon of DMH treated rats for both the durations of 10 and 20 weeks treatment. These reduced selenium concentrations might adversely affect the functional activities of the selenoproteins, compromising protection against oxidative stress.

The availability of selenium and the levels of specific selenoproteins might affect cancer risk by influencing the ability of DNA damaging agents to cause genomic instability and mutations. Baliga et al., (2008) have shown transgenic mice that express reduced levels of selenoproteins and previously shown to be more susceptible to pathology associated with cancer development.

In the present study, increase levels of selenium were shown in the colon of combined treatment of DMH and selenium when compared to only DMH treated rats. Several studies have shown lower selenium levels in different cancer (Willett et al., 1983). Additionally, lowered selenium levels are associated with a 4 to 5-fold increased risk of prostate cancer (Jeffrey et al., 2001). Moreover, results obtained from both durations of 10 and 20 weeks study showed the activity of GSH-Px were significantly increased in the group 4 compared to group 2. Further, the observed lower levels of selenium in the colon tissue of dimethylhydrazine
treated rats compared to the normal tissue support the view that selenium has the ability to inhibit the growth of cancer.

**Alkaline Phosphatase (ALP)**

Alkaline phosphatase (ALP), a kind of membrane-bound metalloenzyme, is related to transport process (Van and De, 1994). Its activity is usually low in normal stomach tissue and is reported to increase once the cell is malignantly transformed (Wang *et al.*, 1996). ALP is usually regarded as an important index of differentiation of cancer cells (Kim, 1984). The increased ALP activities has also been used as a marker of cell proliferation and is linked with a higher risk to develop colon cancer by Kamaleeswari *et al.*, (2006).

During the course of present study, we have observed a remarkable increase in the levels of ALP following 10 and 20 weeks of DMH treatment. Lea *et al.*, (2008) reported that in Caco-2 cells, there was evidence for increased differentiation as judged by increased activity of alkaline phosphatase. Colonic carcinogenesis involves a multistep process of genetic mutations that derange signaling pathways controlling cell proliferation and cell death (Vogelstein and Kinzler, 2004). The observed increase in the enzyme activity could be a consequence of enhanced content of enzyme protein which may be due to several reasons, such as increased synthesis of the enzyme; reduced degradation; enzyme activation by DMH or its metabolites; and conversion of zymogen to an active enzyme. Members of the protein kinase C (PKC) family are important regulators of colonocyte growth and differentiation that are altered during neoplastic transformation. Experimental models of chemical carcinogenesis have been widely used to elucidate signaling abnormalities that contribute to colonic malignant
transformation in colon (Pories et al., 1993). Wali et al. (1995) have reported, azoxymethane-induced rat colonic carcinogenesis showed down-regulation of PKC-ε which is important for cellular processes, including proliferation, differentiation, and apoptosis. Thus, increased level of ALP following DMH treatment in the present study can be due to up-regulation of PKC-ε.

A significant decrease in the levels of ALP was observed after selenium treatments to DMH group at the end of 10 and 20 weeks of study. Jia-Guo et al. (2006) also reported that selenium-enriched malt decreased ALP level in hepatocarcinoma in rats. The reduced ALP activity observed upon selenium treatment to DMH treated rats DMH may be due to the antioxidative and antiproliferative potential of selenium.

ALP, a phosphodiesterase is composed of a group of enzymes with a wide substrate specificity to catalyze the hydrolysis of monophosphate esters at alkaline pH with broad specificity towards different structurally related substrates (Duncan and Prasse, 1986).

The actual substrates in vivo are not known but are postulated to be either ethanolamine phosphate or phoshatidylethanolamine (Kachman and Moss, 1976). ALP is found in abundance in osteoclasts in bone, liver, parenchymal cells and bile duct epithelium, where enzyme activity is associated with the transport of phosphate across the cell membrane. During differentiation of CaCo-2 cells (human colon cancer line), a number of brush border membrane associated hydrolyses, are expressed. Among these, the enzyme alkaline phosphatase, is frequently used as a marker of cell differentiation in this cell line (Matsumoto et al., 1990; Hara et al., 1992).
Total and lipid bound sialic acid studies

Serum total sialic acid (TSA) and lipid-bound sialic acid (LSA) levels have been used as laboratory markers in a variety of pathological conditions (Miyazaki et al., 2004; Albuquerque et al., 2004) and is a highly contentious issue for many years.

Marked elevation in serum TSA concentrations have been seen in DMH treated animals at both the durations of 10 and 20 weeks since sialic acid are membrane components and during carcinogenesis increased cellular proliferation takes place therefore it is understandable that during carcinogenesis sialic acid levels may be increase. Selenium is a cofactor for the enzyme glutathione peroxidase and protects cells from oxidative damage. Sutapa Mukhopadhyay et al. (2000) reported that DNA damage caused by certain free radicals could be prevented by GSH. Administration of selenium may protect the glycoprotein levels through its antioxidant property. As an antioxidant, selenium could prevent lipid peroxidation and thus protect against alteration in membrane structure and function (Wiseman, 1996). Inhibition of DMH carcinogenesis by selenium might involve either selenium inhibition of DMH activation or enhancement of carcinogen detoxification. Also selenium may inhibit cellular proliferation. Selenium is an integral component of the selenoenzyme GPx and there is an evidence to suggest that selenodiglutathione, a metabolite of selenite can specifically inhibit the tumor cell growth and may be capable of functioning as anticarcinogen in vivo conditions (Clement, 1998; Naithani et al., 2008). Thus selenite administration may increase the various seleno-compounds that play important roles in regulating the integrity of cell membranes.
Lipid peroxidation

Free radicals and endogenous peroxides are generated as a result of peroxidation of polyunsaturated fatty acids. The extent of lipid peroxidation was estimated by measuring the levels of malondialdehyde (MDA), a lipid peroxidation product generated in tissue by free radical injury which is measured as a sensitive index of free radical generation (Ichikawa et al., 1999). Oxidative stress pathologies have been linked to lipid peroxidation (Clavel et al., 1985). The increased lipid peroxidation may enhance the oxidative stress in the colon.

Oxidative stress, which is characterized as an imbalance between free radicals and antioxidants in favour of radicals, participates in the pathogenesis of many diseases and their complications (Bankson et al., 1993). Reactive oxygen species (ROS) consisting mainly of superoxide, hydrogen peroxide and hydroxyl radical, have been conventionally considered to have carcinogenic potential (Ames, 1988) and to promote invasiveness (Shinkai et al., 1986). The levels of ROS are controlled by antioxidant enzymes, such as superoxide dismutase (SOD), glutathione peroxidise GSH-Px). Serum malondialdehyde (MDA) level as a marker for lipid peroxidation is commonly used as an indicator for oxidative damage in cells and tissues (Mano et al., 1997). The balance between the formation and removal of lipid peroxides determines the peroxide level in cells. This balance can be disturbed if cellular defences are decreased or if there is a significant increase in peroxidative reactions (Karbownik and Lewnski, 2003).

Formation of reactive oxygen species is a normal consequence of a variety of essential biochemical reactions. It is also known that oxygen radicals could be formed in excess in chronic diseases of the gastrointestinal tract (Girgin et al., 2000). The main source of oxidants is probably phagocytes, which are accumulated in the mucus of patients with bowel diseases and could generate oxidants upon
activation, which might contribute to the increased risk of cancer (Wiseman and Halliwell, 1996).

Oxygen radical production, which increases with clinical progression of diseases, involves increased lipid peroxidation, as a result of which there are cellular membrane degeneration and DNA damage. Extent of lipid peroxidation could be determined by estimation of the final lipid peroxidation products - malondialdehyde and 4-hydroxynonenal, compounds known to produce protein cross-linking through Schiff's base with DNA and DNA damage (Sharma et al., 2001). It has been reported that malondialdehyde is a well-characterized mutagen (Esterbauer et al., 1990) that reacts with deoxyguanosine to form a major endogenous adduct with DNA in human livers (Chaudbary et al., 1994). DNA from colon biopsies had also a significantly increased level of 8-OHdG, 2-hydroxyadenine and 8-hydroxyadenine (Wiseman and Halliwell, 1996). These lesions caused by hydroxyl radical attack could signify the increase in DNA damage and/or decrease in their repair. Moreover, reaction of aldehydes produced during lipid peroxidation with amino acid residues of proteins might lead to their oxidative modifications (Bosch-Morell et al., 1999). In this process, the final products of lipid peroxidation, such as malondialdehyde and 4-hydroxynonenal as well as other products resulting from polyunsaturated fatty acid damage could cause protein breakdown (Esterbauer et al., 1991).

Drastic increase in the lipid peroxidation was observed in the colon of animals following 10 weeks of DMH treatment. There are large number of reports that show increase in MDA concentrations in plasma and tissue of colorectal cancer patients (Hendrickse et al, 1994, Skrzydlewska et al, 2001). It has been claimed that MDA acts as a tumor promoter and co-carcinogenic agent because of its high cytotoxicity and inhibitory action on protective enzymes (Seven et al,
Further, increased serum MDA levels were reported (Huang et al., 1999) in breast cancer patients. Seven et al. (1999) and Samir and Kohly (1999), in their study on patients with laryngeal carcinoma also reported increased MDA levels compared to healthy controls. Pasupathi et al. (2009) reported that increased level of lipid peroxidation and possible breakdown of antioxidant status in cigarette smoking, may increase the risk of gastric cancer. This increase can be explained on the basis of decreased levels of glutathione peroxidase, the enzyme that catalyses the degradation of lipid peroxides as well as hydrogen peroxides. Alternatively, selenium can increase the expression of GPx by stimulation at the genetic level thereby decreasing the MDA levels.

As results have shown, the MDA levels were decreased in selenium supplemented animals which can be explained on the basis of the role of selenium in regulating the glutathione peroxidase activity since selenium is an essential component of glutathione peroxidase. Increase in glutathione peroxidase and other antioxidant enzymes (eg, superoxide dismutases, catalase, and glutathione reductase) tend to reduce lipid peroxidation.

At the end of 20 weeks study, however, the MDA levels showed a significant decrease in DMH treated animals. Manju et al. (2005) reported a significant decrease in lipid peroxidation levels following 15 days of DMH treatment. On the other hand, Gerber et al. (1997) reported decreased MDA levels with increasing tumor size and progression in breast cancer. There are controversial results with regard to MDA levels in cancerous conditions. Several studies have shown reduced rates of lipid peroxidation in the tumor tissue of various types of cancer (Tanaka, 1997; Tanaka et al., 1998; Cheesman et al., 1986; Manju et al., 2005). Increased cell proliferation is thought to be involved in the pathogenesis of colon cancer. Cancer cells tend to proliferate faster when the lipid peroxidation
level is low (Věra Králová et al., 2009). Therefore, the decreased colon and intestinal lipid peroxidation observed in DMH-treated rats could be due to increased cell proliferation at 20 weeks. In addition to this, the decreased levels of lipid peroxidation in DMH-treated rats may also be due to increased resistance and/or decreased susceptibility of the target organs to free radical attack since malignant tissues have been reported to be less susceptible and more resistant to free radical attack.

Selenium administration to DMH-treated rats restored the lipid peroxidation levels to near those of the control rats, which may be due to the antiproliferative activity of selenium (Vasundara et al., 2002). Since proliferation and lipid peroxidation are inversely related (Schmelz et al., 2000) selenium could contribute to the observed increase in lipid peroxidation in the colon. This could increase the susceptibility and decrease the resistance of tumor cells to free radical attack, leading to decreased cell proliferation. Thus, the antiproliferative effect of selenium may be responsible for preventing DMH induced colon cancer.

**Reduced and oxidized glutathione (GSH/GSSG)**

Glutathione is a tripeptide that exist in the thiol reduced (GSH) and disulfide oxidized (GSSG) forms. It is the key component of an endogenous protective mechanism, the glutathione redox cycle against the various xenobiotics compounds (Reed, 1986). It plays a critical role in the detoxification by acting both as a nucleophillic scavenger of various undesired compounds and their toxic metabolites (Reed and Fariss, 1984; Moldeus and Quanguan, 1987) as well as a specific substrate for GSH-Px (Moldeus and Quanguan, 1987) and glutathione-S-transfrase (Arias and Jakoby, 1976). GSH is also known to react readily with a wide variety of free radical species, leading to the formation of the superoxide anions (Munday
Oxidized glutathione (GSSG) is generated from the oxidation of GSH mediated by GSH-Px as follows:

\[
2 \text{GSH} + \text{H}_2\text{O}_2 \rightarrow \text{GSSG} + 2\text{H}_2\text{O}
\]

This reaction requires adequate amount of GSH and selenium (Meister and Anderson, 1983). GSH oxidation can also take place by nonenzymatic transhydrogenation between GSH and cystinyl-bis-glycine which is a product of GSH catabolism (Griffith and Tate, 1980).

Maintenance of GSH/GSSG ratio is considered very crucial to the normal functioning of the cell. Sufficient levels of GSH are thus maintained in a cell by reduction of GSSG to GSH which is catalyzed by glutathione reductase, lipoamide dehydrogenase and thioredoxin reductase (Ziegler, 1985).

In the present study, total glutathione and redox ratio were decreased but the oxidized glutathione levels were increased significantly after 10 weeks of study. Decrease in total glutathione level, as seen in present study can be due to decrease in the levels of GSH in DMH treated rats at the end of 10 weeks. GSH is known to play a major defense mechanistic role against the oxidative attacks (Younes and Siegers, 1981). Thangaiyan and Anupam (2009) reported the exposure of docetaxel with d-limonene makes H_2O_2-scavenging systems break down more rapidly, which is typically observed with the decrease of GSH amount. Decrease in the level of GSH can be explained with the increase in lipid peroxidation at the end of the 10 weeks study. Moreover, these changes were accompanied by a decrease in the levels of glutathione-s-transferase. Another plausible explanation for the reduced GSH levels could be its increased consumption in the removal of peroxides and xenobiotics and low turnover (Meister, 1984). ROS such as H_2O_2 easily oxidizes...
GSH and phospholipids, as well as DNA and proteins, with the diffusion rate and the concentration of targets being the principal limiting step in regulating its activity (Sara et al., 2009). So, the increase in the level of the oxidized GSH may be explained with the increase in the level of ROS. Moreover, significantly increased levels of GSSG were observed in the DMH treated rats which can not be due to the glutathione peroxidase activity, that itself was significantly deceased in this group. Meister and Anderson, (1983) have suggested the oxidation of GSH by nonenzymatic GSH oxidation, which may take place during tranhydrogenation between GSH and cystinyl- bis- glycine which is a product of GSH catabolism (Griffith And Tate, 1980).

On the contrary, the levels of GSH, total glutathione and redox ratio in the DMH treated rats were increased significantly after 20 weeks of study. Vaiyapuri et al. (2005) also reported that chronic DMH treatment results in increased tissue GSH content. Over expression of GSH has been reported in both animal and human tumors by Rajneesh et al.(2008). It has been observed that oxidative stress can cause upregulation of antioxidant enzymes that render cells more resistant to subsequent oxidative insult (Halliwell, 2000).

It is also indicated that GSH-Px levels which are decreased in the DMH group are not sufficient to maintain the glutathione redox cycle at end of the studies. Moreover, the altered levels of lipid peroxidation for two durations of 10 and 20 weeks studies can explain the altered levels of total glutathione and redox ratio. Enhanced levels of GSSG were also found in the DMH treated rats at the end of 20 weeks study. This could be explained on the basis of interaction between selenite and GSH to form a covalent adduct, GS-Se-GS (Shen et al., 1992) which is a central metabolite of inorganic selenium compounds, is further reduced by glutathione reductase (Ganter et al, 1973) or thioredoxine system, leading to the
formation of selenide, which may redox cycle with oxygen leading to the oxidation of thiols. Conversely the increase requirement of GSSG necessitated for maintaining increased level of GSH in order to combat the oxidative stress which is achieved by increased expression of gene.

Supplementation of selenium along with DMH regulated the levels of GSH, GSSG, total glutathione and redox ratio for two durations of 10 and 20 weeks. Since, tissue GSH-Px levels were also increased in the combined DMH and selenium treated animals, supplementation of selenium along with DMH probably maintained the redox cycle indicating utilization of GSH. This might help to combat the free radicals or oxidative stress damage associated with DMH. Further, selenium alters the levels of lipid peroxidation during treatment to the DMH treated animals which further substantiates our finding.

Glutathione-S-Transfrase (GST)

Chemopreventive agents can function by a variety of mechanisms, directed at all major stages of carcinogenesis. Many inhibitors of carcinogenesis have been found to induce phase II detoxification enzymes, including members of the glutathione S-transferase (GST) family (Talalay et al., 1995). Phase II enzymes detoxify electrophilic carcinogens by changing them into a form that is relatively inert and more easily excreted (Talalay et al., 1987). GST detoxifies carcinogenic electrophiles by catalyzing their conjugation with reduced glutathione (GSH) (Chasseaud, 1972; Cho et al., 2007). Glutathione-s transfrases (GSTs) form a group of enzymes that are present in high concentrations in the cytosol and catalyze a wide variety of substitution reactions in which glutathione (GSH) replaces an easily displaced group on the xenobiotic and thus prevents the subsequent toxic reactions (Siddiqui et al., 1990). This reaction involves a
compound with an electrophilic atom and GST facilitates the nucleophilic attack of glutathione thiolate on this electron deficient atom of the hydrophobic compound.

Phase-II reactions in the metabolic degradation of the xenobiotics are chiefly biosynthetic and intend to convert the xenobiotic into a more polar and hydrophilic form to be essentially excreted out of the body and is principally achieved through Glutathione -S- Transfrases.

In the course of current investigations, a statistically significant inhibition in the GST activity was noticed in the animals, which received DMH treatment for two durations of 10 and 20 weeks. Furthermore, animals which were supplemented with selenium along with DMH treatment responded by increased GST activity as compared with their respective DMH treated rat group following 10 and 20 weeks of study.

This enzyme is mainly involved in the free radical scavenging, peroxide reduction and detoxification of xenobiotics through the formation of GSH-S-conjugates (Sies and ketterer, 1988). The electrophilic metabolite conjugates with GSH resulting in the formation of water soluble mercapturic acid which is less toxic than the un-conjugated compounds (Mongenstern et al., 1989). CAT and GST are detoxification/ biotransformation enzymes which are involved with detoxification of toxic substances such as xenobiotics, carcinogens, free radicals and peroxides by conjugating their substances with GSH.

The ultimate carcinogenic form of DMH is a toxic electrophile (methyl diazonium ion and carbonium ion) (Eunju PARK et al., 2007) but GPx and GST take considerable significance in promoting carcinogen detoxification (Beckett and Hayes, 1993). The decrease in GPx and GST activities in colon of tumor-bearing rats may be due to their rapid utilization in detoxification of carcinogenic metabolites of DMH as a part of the body’s defense. Thiyagarajan Devasena et al.
(2006) earlier reported that GST levels decreased in the RBC lysate of tumor-bearing rats with DMH treatments.

Administration of selenium to DMH-treated rats altered the levels of LPO and increased the activities of GPx and GST in the circulation. Our findings are also in line with the views of Van Leishout and Peters (2000) that inhibitors of carcinogenesis have an enhancing effect on carcinogen detoxifying enzymes. GPx and GST enhances the biotransformation of carcinogens (Hayes and Pulford, 1995). Induction of enzymes that are involved in the biotransformation of carcinogens may accelerate the metabolic disposal of carcinogens (Devasena et al., 2003; Hayes and Pulford, 1995; Szatrowski and Nathan, 1991). Raymond et al. (2004) suggested that garlic consumption prevents AFB1 carcinogenicity by favoring the formation of AFBO–glutathione conjugate through up-regulation of GST.

**Superoxide dismutase (SOD)**

The reactive oxygen species are generated during normal metabolism and cells contain multiple protective systems, which limit their damaging effects. These include net work of protective enzymes and antioxidants, which prevent or intervene in the injurious oxidative reaction initiated by these species. The first line of defense against superoxide is superoxide dismutase. Superoxide dismutase (SOD) catalyzes the dismutation of superoxide anion ($O_2^-$) to hydrogen peroxide ($H_2O_2$) in cell cytosol and mitochondria (Salin and McCord, 1974). Because the mitochondrial electron transport system produces endogenous $O_2^-$ (Loschen et al., 1974), and SOD has been suggested to play a crucial role in the protection of the organelle from oxidative damage (Slot et al., 1986).
In the present study, SOD activity was found to be decreased in the colon of DMH group animals at the end of 10 and 20 weeks study. Decreased level of SOD has been reported earlier in various malignancies (Rao et al., 2000). The decreased activities of SOD can also be attributed to resistance offered by the animals in mitigating the toxic stress on the body. The decreased activity of SOD in DMH treated rats may have been due to the enhanced lipid peroxidation or inactivation of the antioxidative enzymes. This may have caused an increased accumulation of superoxide radicals, which could have further stimulated lipid peroxidation (Park et al. 2001). SOD plays a vital role in the detoxifications of reactive oxygen species. The low levels of antioxidants could be as a result of this increased oxidative damage; or it could be that low values aggravated the free radical damage and increased the chance of developing cancer, indicating antioxidants role in prevention and role of oxidative injury in the causation of cancer. H$_2$O$_2$, the end product of SOD, is the substrate of glutathione peroxidase and catalase which catalyses to H$_2$O and O$_2$. H$_2$O$_2$ is known to inactivate SOD (Bray et al., 1974; Demopoulos et al., 1980). Since, the level of glutathione peroxidase decreased in DMH treated animals and resulted in an increase in the levels of H$_2$O$_2$ and hence the inhibition of SOD activity.

Moreover, Selenium supplementation to the DMH treated animals resulted in a significant increase in the SOD and GSH-Px levels that generate a condition where H$_2$O$_2$ is not available for inhibiting SOD, by increasing GSH-Px levels, which in turn increases SOD levels to remove O$_2^-$ radical from system for two duration of 10 and 20 weeks study.
Catalase

Catalase is present in the peroxisomes of mammalian cells, and probably serves to destroy H$_2$O$_2$ generated by oxidase enzymes located within these subcellular organelles. Catalase is responsible for the decomposition of hydrogen peroxide. Catalase forms an integral component of cellular antioxidant defense mechanism. This enzyme is primarily responsible for the removal of H$_2$O$_2$ from the cell. Hydrogen peroxide is a normal cellular metabolite (Singer and Edmondson, 1974) produced by the action of superoxide dismutase during the conversion of the reactive form of oxygen to less reactive forms. This is also produced during the oxidative damage to the tissues. It is formed in all the subcellular compartments of the cell (Boveris et al., 1972). Therefore, the management of removal of H$_2$O$_2$ simultaneously with the production is of immense importance. Cohen and Hochestein, (1963) and Nicholls, (1972) also demonstrated that in the erythrocytes, glutathione peroxidase played a principal role at the low concentrations of H$_2$O$_2$ whereas at the higher rates of H$_2$O$_2$ generation, role of catalase was more important. The induction of the motility loss resulting from the action of ROS and specifically hydrogen peroxide was ameliorated by the action of catalase (Macleod, 1943).

In the present study, catalase activity was found to be significantly decreased in the colons of animals subjected to DMH treatments for 10 and 20 weeks. Decreased activities of SOD and CAT in DMH treated rats is in agreement with other reported studies by Safinaz and Noha, (2008) and Sundaesran and Subramanian (2003). The decrease in catalase activity may be due to over-utilization of these enzymatic antioxidants to scavenge the products of lipid peroxidation. Increased level of O$_2^-$ can also inactivate catalase and lead to the accumulation of H$_2$O$_2$ levels in the tissue. ROS finally give rise to superoxide
anion radical $O_2^-$ (Osthoff et al., 1992). Enhanced levels of $O_2^-$ are able to convert the catalase to the ferroxy catalase (Fridovich, 1982) or ferryl state (Kono and Fridovich, 1982) which is inactive form of the enzyme. Further, Burton et al. (1983) have reported that some human cancer cell lines produce large amounts of $H_2O_2$. Therefore, decrease in the levels of catalase could possibly be due to utilization of this enzyme in converting the $H_2O_2$ to $H_2O$. In recent years, several groups have focused their studies on structural protein modifications by free radicals (Davies et al., 1987; Du and Gebicki, 2002). Recently it has been demonstrated that superoxide anion as well as alkoxyl peroxyl and radicals could inactivate catalase and reduce the effectiveness of cells to defend against free radical damage (Mayo et al., 2003).

A significant increase in the catalase activity has been observed in the selenium supplemented group to the DMH treated rats at the end of 10 and 20 weeks treatments. Qun et al., (2009) observed activity of CAT can be decreased in chronic fluoride exposure, whereas selenium can inhibit the effect of fluoride on antioxidant enzyme activities and increase the activities.

This can be explained with the increased levels of SOD which results in the decreased levels of $O_2^-$. 

**Glutathione Reductase**

Glutathione reductase (GR), a flavoprotein (FAD containing enzyme), catalyses the regeneration of reduced glutathione.

$$\text{GSSG} + \text{NADPH} + H^+ \xrightarrow{\text{GR}} 2\text{GSH} + \text{NADP}^+$$
Discussion

This enzyme requires NADPH as the source of reducing power which is generated by hexose monophosphate pathway.

Glutathione reductase seems to work in unison with glutathione peroxides, which is normally responsible for the oxidation of GSH. Therefore this enzyme plays an important role in maintaining redox status of the cell.

In the present study, activity of GR was found to be enhanced in the DMH group after both treatment periods. In support of the present result, Manju et al., (2005) have reported that level of GR was increased in the DMH group. Sahdeo et al., (2008) also reported the increased level of GR in androgen induced oxidative stress. Eggleston and Krebs, (1974) have shown that the activity of enzyme was regulated by both NADPH and GSSG levels. It was also noted that the high concentration of oxidized glutathione can even reactive the enzyme. Moreover, Rushmore et al., (1991) has reported that increase in the activity of glutathione reductase might occur as a result of induction of genetic expression. At the same time, oxygen radicals might increase secretion of the matrix metalloproteinase and collagenase as well as production of angiogenic factors (e.g. VEGF and IL-8). These factors could promote not only the local growth of neoplasm but also metastasis (Brown et al., 2000). SOD converts superoxide radicals into hydrogen peroxide, which in turn has to be removed by CAT and GR. This is in agreement with the results of our present study since the increase activity of GR and decreased levels of SOD were observed in DMH treated rats. Further, when the level of GSH are low as seen in DMH treated animals, the feedback comes in operation in order to combat the stress and as a consequence thereof the enzyme GR gets over expressed in order to increase GSH.

Selenium supplementation to the DMH group probably decreased the level of oxygen radicals by improving SOD activity.
Glutathione peroxidase

Glutathione peroxidase, a selenium dependant enzyme, is a cytosolic protein responsible for the removal of H$_2$O$_2$ and organic peroxides which requires GSH for its activity (De Duve and Baudhuin, 1966).

In the present study, a significant decrease in the GSH-Px activity was seen in the colon tissue of DMH treated animals as compared to controls animals for two durations of 10 and 20 weeks. Present results are supported by findings of Aranganathan et al., (2008) which show were decreased activities of GPx in the DMH treated group. Durak et al. (1996) showed that GSH-Px activities were lower in carcinomas. The decreased GSH content and a fall in the activities of GPx and GST were noticed in RBCs of DMH treated rats (Thiyagarajan Devasena et al., 2006). This is in accordance with our finding that the GSH levels get decreased following 10 weeks of DMH treatment. Therefore, the deficiency of GSH in DMH treated rats as observed in our study may explain the decreased activity of glutathione peroxidase. On the contrary, the glutathione peroxidase activities were found to be decreased, while the GSH levels was enhanced in DMH treated rats during 20 weeks study. This may be due to the increased utilization of GPx in detoxification of carcinogenic metabolites of DMH.

Selenium supplementation to DMH treated animals shows a significant increase in the GSH-Px activity at the end of both durations of 10 and 20 weeks. Sunde and Evenson, (1987) were reported that selenium is required in the active site of glutathione peroxidase which plays an important role in maintaining the redox cycle in the cell. Zeng et al.(2009) reported that Methylselenol, a selenium metabolite, induced cell cycle arrest in G1 phase and apoptosis via the extracellular-regulated kinase 1/2 pathway and other cancer signaling genes which may contribute to the inhibition of tumor cell invasion. Antioxidants, including
selenium, protect cells from DNA oxidative damage by scavenging free radicals in epithelial cells of the breast that can cause cancer (Borek, 2004; Key et al., 2003).

**FT-IR studies**

Fourier-transform infrared (FT-IR) spectroscopy can effectively provide chemical variation information of the structure and composition of biologic materials at molecular level (Wong et al., 1991). Therefore, vibrational spectroscopy i.e. FTIR is becoming an increasingly powerful tool for the research on biochemistry of cancer (Fujioka et al., 2004; Wong et al., 1995; Sindhuphak et al., 2003; Argov et al., 2002). Several researchers have successfully used FTIR spectroscopy to diagnose carcinomas, such as carcinoma of stomach, colon, esophagus, lung, salivary gland (Ling et al., 2002; Li et al., 2004; Ren et al., 2003; Sun et al., 1996). There are significant differences between the spectra of malignant and corresponding normal tissues due to variation in the structure and composition of chemical groups (Wu et al., 2001; Xu et al., 2000). In addition, FTIR spectroscopy could detect molecular abnormalities which occur before the change in morphology seen under light microscope (Zhang et al., 2004). Significant differences between normal and DMH treated tissues were seen in FT-IR spectra in this study. They might be caused by the changes of content and space array of protein, nuclear acid, sugar and fat in cells. The spectral changes so observed in the present study following DMH treatment reflects alterations in the structure of important informational and structural molecules, mainly involving the C—O groups in cell proteins and C═O groups of acyl chains of membrane lipids. Further the packing and the conformational structure of the methylene chains of membrane lipids are changed in colonic tissue treated with DMH. The appearance of peaks in the range of 3000-280 cm⁻¹ may be due to C-H stretching vibrations in
the membrane phospholipids, as observed by the state of lipid peroxidation, the DMH may cause membrane damage which is manifested as change in the spectral behavior.

Although our findings do not allow the more specific assignment of these changes, nevertheless they indicate extensive chemical and physical changes in various macromolecules during the initiation of colon carcinogenesis. Whatever the exact nature of these alterations, the present study shall have a practical bearing and would suggest that determination of these spectra may be of use in rapid and early evaluation of the cancerous phenotype in colonic tissue. Further, these changes can also be used and attributed for the classification purposes as well. Therefore, FT-IR technology makes it possible to detect inflammatory and precancerous changes.

Koeing and tabb (1980) have shown that the secondary structure of the globular proteins can be distinguished by infrared spectroscopy by using a combination of amide I frequency and the intensity of amide III region. The peaks in the range of 1652-1656 cm\(^{-1}\) corresponding to α-helix, near 1632 cm\(^{-1}\) corresponding to β-sheet and near 1655 cm\(^{-1}\) corresponding to disordered state. Thus, we can clearly see a marked changes in the secondary structure of the membranous proteins which can finally lead to changes in the conformation of protein and hence it biological function. Richard A. Dluhy et al., (1988) concluded that absorbance bands in the 3000-280cm\(^{-1}\) arises primarily due to C-H stretching vibrations of phospholipids acyl chains. Selenium supplementation for 10 weeks and 20 weeks to DMH treated rat resulted in the appearance and disappearance of a new peaks. Selenium has been shown to have pronounced effects on the physical state of the membrane. Wu et al., (1979) also have shown that selenium has
important role in maintaining the integrity of the sperm membrane by its selenium depend-enzyme glutathione peroxidase. Noguchi et al. (1973) proposed that the membrane protecting effect of Se was one of preventing oxidative damage to the membrane lipids.

**Physical states of membrane**

The luminal (brush border) and contraluminal (basolateral) plasma membranes of enterocytes and colonocytes, the predominant cells lining the small and large intestine respectively, are highly specialized to perform certain physiological functions (Petter et al., 2009). These antipodal membranes in both cell types, which function to regulate exchange between organism and environment, also differ from each other in a number of respects (Petter et al., 2009), including lipid composition and fluidity (Kakimoto et al., 1995). Moreover, considerable evidence exists that alterations in the lipid fluidity of these membranes can influence many important functions of these cells, including the activities of certain enzymes such as alkaline phosphatase (Brasitus and Dudeja, 1985).

The brush border membrane (BBM) comes into direct contact with the material present in the intestine to be absorbed. One important way can be studied is by looking with transitional diffusion of pyrene and its excimer formation in the membrane (Massey et al., 1982). Changes in the membrane lipid composition are also important indicators of membrane fluidity (Ghosh and Mukherjee, 1995).

The mucosa and BBM of the gastrointestinal tract, called the GI barrier, protects the intestinal lumen from the toxins (Gisolfi, 2000) and comes in direct contact with the material present in the intestine and mostly affected by the carcinogenic drugs. In the present study, results showed significant increase in the E/M ratios as
Discussion

compared to the control group following 10 weeks of DMH treatment. The increased E/M ratio or increased lateral diffusion of the probe (pyrene) indicates a decrease in membrane microviscosity, which further suggests, an elevation of membrane fluidity. The increased E/M ratio or increased lateral diffusion of the probe i.e. pyrene in the membrane might have resulted due to partial lipid removal and more motional freedom of the probe in the hydrocarbon phase. This has been reported earlier that increased excimer formation is indicative of enhanced fluidity of the membrane and the enhanced translational or lateral mobility of the probe in the bilayer (Vanderkooi and Callis, 1974; Galla and Sackmann, 1974).

Alterations in the lipid or protein composition may change the membrane fluidity, which is determined by lipid-protein interactions and in membrane fluidity is directly linked with membrane functions (Brasitus and Schacter, 1980). Lipid profile and membrane fluidity has been observed to be altered in various pathophysiological conditions, and therefore, is an important index of cellular integrity. An increase in lipid content may also indicate an increase in fluidity across the intestinal membrane (Proulx, 1991). The increased lateral diffusion of the probe might be a result of partial lipid removal and more motional freedom of the probe in the hydrocarbon phase.

Many factors could account for the observed increase in membrane fluidity and polarization of the probe during development of experimentally induced colon carcinogenesis. One possible explanation could be the peroxidation of membrane phospholipids induced by reactive oxygen species released during the initiation of cancer as observed after 10 weeks of DMH treatment. In addition, pyrene diffusion and excimer formation may be hampered by the inhomogeneity of the membrane. Simultaneous supplementation of selenium for 10 weeks to DMH treated rats significantly decreased the E/M ratio, when compared to DMH alone treated
group, suggesting a decrease in membrane fluidity. Selenium has been shown to have pronounced effects on the physical state of the membrane.

Selenium's primary function in the animal body is as a component of an enzyme called glutathione peroxidase. This enzyme is important in protecting the integrity of cell membranes. During normal metabolism, the cell produces reactive forms of oxygen (peroxides) which, if not altered, will damage the unsaturated fatty acids found in the cell membrane. Membrane damage will disrupt cell function and adversely affect animal health. Glutathione peroxidase is found in red and white blood cells, heart muscle, brain, fat, lungs, liver, kidney, and skeletal muscle. Insufficient dietary selenium results in a glutathione peroxidase deficiency throughout the body.

It is believed that the damage of immune cells caused by lack of the minor elements of selenium, proliferation of free particles and peroxidization of lipid are probably associated with cell immune functional disorders in patients with chronic hepatitis (Yu et al., 1999; Loguercio et al., 2001; Hatano et al., 2000). Fluidity, one of the basic characteristics of the membranes, is the basic precondition of cells showing various functions. In many pathologic cases, excessive free particles and initiating lipoperoxide could affect membrane fluidity and lead to disorder of cell immune functions (Gomicki and Gutsze, 2001(a); Ferrante et al., 2002; Garcia et al., 2001; Gomicki and Gutsze, 2001(b)). Selenium and enzymes in combination with selenium can inhibit cell membrane peroxidation damage and defend membrane fluidity as well as functional expression. Further, decreased membrane fluidity following 10 weeks selenium treatment to DMH treated rat could also be indirectly attributed to the regulatory role of selenium in maintaining the ROS levels in the cell.
On the contrary, DMH treatment for 20 weeks showed a decrease in E/M ratio as compared to normal control group. Further, a significant decrease in the fluorescence polarization was observed following 20 weeks of DMH treatment, indicating an increased resistant environment for the probe and thus, a decreased fluidity of the membrane during the post initiation stages of colon carcinogenesis. Considerable evidence exists that many functions of biological membranes are influenced by composition and physical state of membrane lipids (Silvius, 1982). Recently, a report by Nalini et al. (2006) has also shown changes in the phospholipids (phosphatidylinositol, phosphatidylserine, phosphatidylethanolamine, phosphatidyl-choline) content in the cell membranes of long term DMH treated colons, suggesting that the decreased membrane fluidity as observed in the present study in the colons subjected to long term DMH treatment could be due, at least in part, to an altered phospholipid composition. Supplementation of selenium to DMH treated rats also revealed a significant decrease in membrane fluidity when compared to control rats.

**Histoarchitectural studies**

In the present study, histological and ultrastructural changes were examined in colon tissues of rats belonging to different treatment groups.

**Scanning electron microscopic studies**

Results of SEM studies confirm the results of light microscopic studies. Luminal surface of the DMH treated rats showed the orifice of protuberant gland units was slit-like rather than round and funnel-shaped gland units having surface absorptive cells more convex, occasional mucous, malignant cells were seen
between absorptive and mucous cells following 10 weeks of treatment. Moreover, it was seen that 2 to 4 smaller glands were present in the deeper layers. We observed further morphological changes during period of 20 weeks where tumors were observed in the colonic epithelium. The SEM showed malignant surface cells were observed crater-like area of small plaques and revealed them to be clearly different in appearance from cells seen previously. David et al., (1977) have reported as the duration of treatment increased, normal gland units were progressively replaced with enlarged gland units, and 26 weeks after treatment the entire mucosa was abnormal in appearance. In our study, the typical, funnel-shaped glands seen adjacent to DMH-induced tumors appear similar to that reported in some specimens of human colonic epithelium obtained from biopsy samples of tumor-bearing patients (Fenoglio, et al., 1975). In DMH-treated animals, it has been shown that cell numbers in the intestinal glands are increased (Tutton, and Barkla, 1976) and that the rate of cecal tumor formation is increased at sites of suture induced chronic inflammation (Pozharisski, 1975). These findings and the findings of the present work suggest that morphological change precede tumor formation in DMH-treated animals and may indicate that the modality of DMH treatment is to progressively after the factors influencing cell proliferation in normal animals to such an extent that some local, nonspecific stimulus to cell proliferation, such as suture placement, can trigger a cell into undergoing malignant change. Goblet cells, meanwhile, elaborate mucins, trefoil proteins and other factors that help protect the intestinal mucosa from injury and facilitate tissue repair (Deplancke and Gaskins, 2001). Thus, reduction in the number of goblet cells cause a reduction in the total amount of mucous produced, making the colonic surface more prone to injuries (Hasse et al, 1973). Further, the surface epithelial elevation might be due to hyper-proliferative activity of colonic cells following
carcinogen treatment. Eleanor and Deschner, 1978 have also reported an increase in cell proliferation in upper crypt of the DMH treated rat colon.

**Light microscopic studies**

The histopathological findings at the light microscopic level revealed a disturbed histoarchitecture of colons following 10 weeks of DMH treatment. The colon tissue sections from DMH treated rats show the occurrence of disorganization of colonic histoarchitecture following 10 weeks of treatment. The epithelial cells showed a loss in nuclear polarity and enlargement of epithelial cells with mild inflammation of lamina propria. However, no signs of hyperplasia/dysplasia were observed. In the combined treatment group (Group IV), histoarchitecture revealed restoration of the near normal architecture of the colons following 10 weeks of selenium supplementation to DMH treated rats. The size and shape of the cells were uniform with mild nuclear enlargement. Histoarchitectural changes in the colons can be explained on the basis of alterations in the lipid composition and integrity of membranes in response to DMH treatment.

Sections from DMH treated groups, showed the well differentiated signs of hyperplasia/ dysplasia followed 20 weeks of study. Nuclei were enlarged, deeply stained of epithelium, dysplastic crypt lacking mucin and cells were hyperchromatic, increased mitotic activity and loss in nuclear polarity. Further, tumor sections from colons of DMH treated rats showed invasive tumor cells and formation of signet ring cell carcinoma indicating the malignant transformation occurring in the epithelial surface. Similar result have earlier been reported from our lab (Dhawan *et al.*, 2006) whereby, DMH treatment for a period of 16 weeks
clearly showed well differentiated sighs of dysplasia which also was greatly restored when zinc supplementation.

Cancers of the large intestine in the animal model by chemical carcinogen resemble adenocarcinomas in humans, and spread in a similar manner. Evidence from animal studies has shown that experimental colonic tumours induced by procarcinogen 1, 2- dimethylhydrazine (DMH) are of epithelial origin with a similar histology, morphology and anatomy to human colonic neoplasms (Maskens, 1976). Furthermore, prior to the development of colonic cancer, injections of DMH could result in increased colonic crypt cellularity, colonic crypt cell proliferation and colonic crypt proliferative zone as observed in the 10 weeks treatment group (Richards, 1977). Several studies showed that DMH-induced adenocarcinomas are similar to those of human colon carcinoma (Shamsuddin and Trump, 1981). Animal studies support epidemiological and human experimental observations of dietary factors involved in colorectal cancer formation.

Histoarchitecture of animals subjected to selenium treatment with DMH revealed no signs of dysplasia but indicated a little loss of nuclear polarity. Our observations clearly suggested the significant effect of selenium in containing the DMH induced histological changes in colon tumors, which can greatly affect the post initiation stages of colon carcinogenesis by altering the efficacy at which DMH can initiate histological changes.

**Transmission electron microscopic studies**

Transmission electron microscopic (TEM) technique was used to see the changes in colon cells at ultrastructural level. Most exciting changes associated with DMH treated group observed were prominent effects on the mitochondria, endoplasmic reticulum and cellular integrity. Another prominent feature of the
present study was an increase in the number of mitochondria which indicated and increased energy requirement of the cells in an effort to overcome the increased demand for the energy to be needed by various biosynthetic pathways required for DNA and protein synthesis. As regards the expansion of reticular endoplasmic reticulum (RER) that gets substantiated with the increased demand of the protein synthesis and seems be the only plausible reason. As has already been discussed in detail about the effect of DMH on free radical generation, which has a direct bearing on the membrane lipids and ultimately on the membranes of ER and the same. This further initiates a chain of events, due to which body requirements of energy are increased and have direct bearing on the mitochondrial and nuclear structures. Selenium supplementation to DMH treated rats resulted in ultrastructural changes with regard to integrity of the cells as a whole as well as the cell organelles get affected by DMH treatment. This may explained by role of selenium in improving function of mitochondria by preventing free radical damage.

**Micronucleus assay**

The oxidation of biomolecules due to reactive oxygen species (ROS) is associated with cellular dysfunction, and leads to various biological responses such as inflammation and apoptosis. When ROS attack DNA, oxidized bases are generated and the un repaired oxidative DNA damage can induce mutations. Formation of hydroxylated bases of DNA is considered as an important event in chemical carcinogenesis (Breimer 1990, Bartsch H, Nair J 2002). This modification of DNA bases lead to chromosomal aberrations formation of DNA adducts micronuclei formation in the cell (Cerutti, 1994). Kinsella and Radman, (1980) have reported the carcinogenic potencies of physical and chemical agents correlate well with their capacity to damage DNA.
In the present study, a significant increase was observed in micronucleated cell score in DMH treated animals when compared to control group for both durations of 10 and 20 weeks. The increase in score of micronucleated cells indicated that DMH caused DNA damage. DNA damage has been repeated to cause the formation of micronuclei in cells. 1,2-dimethylhydrazine induced micronucleated colonic epithelial cells has been earlier reported in their studies by Ohyama et al., (2002). Selenium supplementation to the DMH treated rats showed a significant decrease in the score of micronucleated cells, which indicated the potential role of selenium to reduce the number of micronucleated cells. Thus, selenium is involved in scavenging of free radicals and ROS and protects cell from chromosomal aberrations and decrease the score of micronucleated cells in colon tissue.