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Full Length Research paper

Protective role of gossypol against N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) induced gastric carcinogenesis in experimental rats

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Gossypol, $C_{30}H_{30}O_8$, is a polyphenolic aldehyde compound derived from the cotton plant (genus *Gossypium*, family Malvaceae) that permeates cells and inhibits wide range of cell growth. It also disturbs a variety of cellular enzymes that are known to be involved in energy production. According to literature survey, there is no study on the effect of gossypol on experimental gastric cancer. Here, we used gossypol as a model of polyphenolic compounds, because it possesses anti-inflammatory and antioxidative properties and is used as a dietary supplement. The animals were divided into five groups and the effects of gossypol on simultaneous and post-treated stages were studied in MNNG induced animals. The results of body and tumor weights were monitored, significant increase in the level of lipid peroxides and protein carbonyls were observed on gossypol treatment. There is also a significant alteration in the antioxidant status which is found to be increased on administration of gossypol at a dosage of 40 mg/kgbwt for 30 days. The results of the present study suggest that gossypol may exert its cytoprotective effects by modulating lipid peroxidation and enhancing the level of antioxidant enzymes status of the tumor bearing animal. Thus, we conclude that up-regulation of antioxidants by gossypol treatment might be responsible for the decreased effect in gastric carcinoma.

Key words: Gossypol, N-methyl-N'-nitro-N-nitrosoguanidine, lipid peroxidation, protein carbonyls, superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glutathione, glucose-6-phosphate dehydrogenase, body weight and tumor weight.

INTRODUCTION

Gastric cancer is a major cause of morbidity and mortality world wide (Gore, 1997). Most cases (85%) of gastric cancer are adenocarcinomas that occur in the lining of the stomach (mucosa). Approximately, 40% of cases develop in the lower part of the stomach (pylorus), 40% develop in the middle part (body) and 15% develop in the upper part (cardia). In about 10% of cases, cancer develops in more than one part of the organ. Multiple environmental factors including chronic *Helicobacter pylori* infection and dietary factors have been implicated in the initiation of gastric carcinogenesis (Nomura et al., 1991; Jossens and Geboerse, 1981). Gastric carcinoma spreads locally by direct invasion through the gastric wall into adjacent tissues and metastasizes to regional lymph-nodes and distant organs through lymphatic and venous vessels. The most common form of gastric cancer is adenocarcinoma (Lewin and Appelman, 1995; Lauren, 1965). N-methyl-N-nitro-N-nitrosoguanidine (MNNG) induced gastric cancer in male albino. Wistar rats show similarities to human gastric tumors (Mirrish, 1975). Humans are exposed to MNNG like carcinogenic compounds through luminal nitrosylation of naturally occurring guanidine compounds such as L-arginine and creatinine by dietary nitrite in the presence of acid in the stomach (Endo et al., 1975; Arivazhagan et al., 2000). A high salt concentration in the stomach destroys the mucosal barrier and leads to inflammation and damage such as diffuse erosion and degeneration. It is therefore biologically plausible that high salt intake increases the risk of gastric cancer in humans.

Oxidation and production of free radicals and reactive oxygen-containing species (ROS) are an integral part of

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life and our metabolism. They are formed as necessary intermediates in a variety of normal biochemical reactions, but when they are generated in excess or not appropriately controlled, these free radicals can wreak havoc on a broad range of macromolecules. Complex antioxidants prevent the oxidative damage by removing or inactivating chemical intermediates that produce free radicals. If antioxidant fails to prevent the oxidative damage then it leads to faulty disposal of free radicals and its accumulation.

These ROS are responsible for oxidation of tissues leading to lipid peroxidation and tissue damage. They are also responsible for oxidation of bases in cellular DNA making them mutagenic cytotoxic and cross linking agents which in turn causes uncontrolled expression of certain genes causing increased multiplication of cells leading to cancer (Fridorich, 1986).

Chemotherapeutic and chemoprevention by synthetic compounds have evolved as a novel approach to control cancer incidence. Medicinal plants and their active principles have received growing attention in recent years as potential chemopreventive agents. The present study focused and evaluated on gastric cancer using gossypol as a chemotherapeutic agent. Gossypol is the source of cotton fiber, cottonseed oil, which is used for cooking. It is currently believed that gossypol in itself will not kill cancerous cells; however, it changes the chemistry within the cancer cell and makes it more susceptible to traditional chemotherapy drugs. Phased trials have been done on resistant prostate and lung cancer. However, no study where done in gastric cancer. Some evidence suggests that gossypol is a potent anticancer drug because of its broad spectrum of inhibitory activity (Wu Chick et al., 1989). *In vivo* studies have demonstrated that gossypol treatment enhanced the survival of nude mice bearing Ehrlich as cites tumor (Tso, 1984) and human adrenal cancer (Flock et al., 1993). But there was no data available on the effect of gossypol on antioxidant enzymes and lipid peroxidation in experimental gastric cancer.

The objective of this study is to evaluate the effects of gossypol on cell membrane damage and antioxidant enzymes in MNNG induced experimental gastric cancer in animal model.

MATERIALS AND METHODS

Animals

Thirty inbred male albino Wistar rats (130 - 150 kg) were used in this study and they were classified into 5 groups of six rats each. The animals were purchased from Central Animal House Facility, Dr. ALM PG IBMS, University of Madras, Taramani, Chennai – 600 113, India and maintained in a controlled environmental condition of temperature (23 ± 2°C) and relative humidity (50 - 70%) on alternatively 12 h light/dark cycles. All animals were fed standard pellet diet (Gold Mohor rat feed, M/s. Hindustan Lever Ltd., Mumbai) and water *ad libitum.* This research work on wistar albino male rats was sanctioned and approved by the institutional animal ethical (IAEC NO. 02/018/08)

Experimental design

The animals were divided into five groups of six rats each.

Group I

Control animals treated with DMSO (vehicle) orally for 25 weeks.

Group II

N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) (200 mg/kgb. wt, by oral gavage) treated at days 0 and 14. Saturated NaCl (1 ml/rat) were given to these rats 2 times per week for 3 weeks and then placed on basal diet for 25 weeks.

Group III

MNNG (as in group II) and gossypol (40 mg/kg b.wt, dissolved in DMSO) treated simultaneously for 25 weeks from the first day of MNNG (as in Group II).

Group IV

Gossypol (as in Group III) post treated from the 16th week of MNNG treatment up to 25th week.

Group V

Control animals treated with gossypol for 30 days.

After the experimental period of 25 weeks, the animals were sacrificed and the parameters like antioxidant and lipid peroxidation assays were determined in the stomach and liver tissues of experimental and control animals.

Lipid peroxidation, evidenced by the formation of thiobarbituric acid reactive substance (TBARS) was assayed in tissue sample as described by Ohkawa et al. (1979). The antioxidant enzyme such as superoxide dismutase (SOD) was determined by the method of Marklund and Marklund (1974), catalase was assayed by the method of Sinha (1972), glutathione peroxidase (Gpx) was assayed by the method of Rotruck et al. (1973), glutathione reductase (GR) by Staal et al. (1969), the level of reduced glutathione was measured by the method of Moron et al. (1979) and glucose-6-phosphate dehydrogenase was assayed by the method Korenberg and Horecker (1995). The data presented as mean ± SD were analyzed using ANOVA.

RESULTS

Table 1 represents the body weight changes of the control and experimental rats. Body weights and tumor weight were noted from the day of tumor induction, till the completion of the experimental period. Weights were noted periodically once in a weak.

The control rats did not show significant change in body weight throughout the experimental period. There was a sharp drop in the body weight and increased tumor weight of the gastric carcinoma bearing rats when compared with the normal control rats. Gossypol treated group III and group IV showed gradual increase in their body weight when compared with cancer bearing group II

Table 1. Effect of gossypol on body and tumor weight of control and experimental animals.

Treatment Groups	Group I: Control alone	Group II: MNNG treated	Group III: Gossypol pretreated	Group IV: Gossypol post treated	Group V: Gossypol alone
Body weight (gm)	304.06± 27.36	145.42 ± 13.08a*	271.12 ± 24.41b*	225.14 ± 20.26c@	312.14 ± 28.01
Tumor weight (gm)	-	0.62 ± 0.07	0.24 ± 0.02a*	0.38 ± 0.04b#	-

Each value is expressed as mean ± SD for six rats in each group.
Body weight: a, as compared with group I; b, as compared with group II; c, as compared with group III. Tumor weight: a, as compared with group II; b, as compared with group III.
Statistical significance- * p < 0.001, @p < 0.01, #p < 0.05.

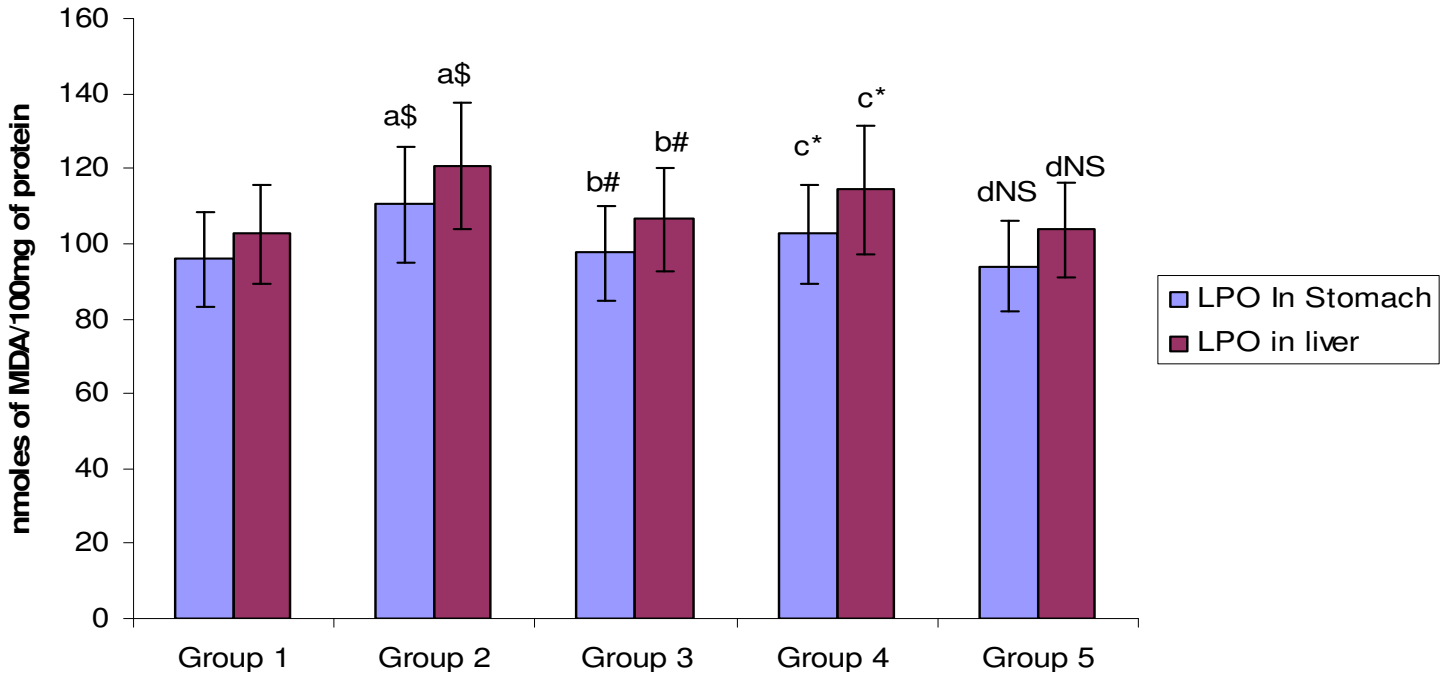

Figure 1. Levels of lipid peroxide in stomach and liver of control and experimental groups. Values are expressed as mean ± SD of six rats each group. a-compared with group I; b-compared with group II; c-compared with induced group II; d-compared with group I; statistical significance - $p < 0.001, #p < 0.01, *p < 0.05; NS-not significant.

animals. Gossypol drug control animals showed an increase in their body weight but were not significant when compared with normal control animals.

Figure 1 and Table 2 show the levels of lipid peroxidation and protein oxidation in stomach and liver of control and experimental rats in each group. In induced rats, the lipid peroxidation and protein oxidation levels have significantly increased when compared to control rats. Whereas pre-treated and post-treated rats show these levels to be statistically (P < 0.001, P < 0.05 and P < 0.01) and significantly decreased when compare to induced rats group II, but in gossypol alone treated rats, these levels near to control rats.

Figures 2 and 3 show the level of antioxidants status in the stomach and liver of control and experimental rats in each group. There is significant reduction in the activity of SOD, catalase and Gpx in cancer bearing rats when compared with control rats and there is significant increase in the activity of SOD, catalase and Gpx in gossypol treated group 3 and group 4 rats cancer (P < 0.001, P < 0.05 and P < 0.01) when compared with cancer bearing rats and the levels of gossypol alone treated rats are significantly closer to control rats.

Tables 3 and 4 show the level of GSH, GR and G6PDH enzymes status in the stomach and liver of control and experimental rats in each group. The levels of glutathione enzymes GSH, GR and G6PDH are significantly decreased in cancer bearing rats, whereas gossypol treatment in group 3 and group 4 caused a significant (P < 0.001, P < 0.05 and P < 0.01) increase in glutathione enzymes when compare with cancer bearing rats, gossypol alone treated rats show no significance when

Table 2. The levels of GSH, GR and G6PDH in stomach of control and experimental animals (Mean±SD, N=6).

Treatment groups	Group I: Control alone	Group II: MNNG treated	Group III: Gossypol pretreated	Group IV: Gossypol post treated	Group V: Gossypol alone
Stomach protein carbonyl (nmoles/mg of proteins)	2.18 ± 0.35	3.81 ± 0.45 $a^{\#}$	2.45 ± 0.29 $b^{\#}$	3.13 ± 0.37 c^{*}	1.93 ± 0.27 d^{NS}
Liver protein carbonyl (nmoles/mg of proteins)	1.85 ± 0.29	3.47 ± 0.53 $a^{\#}$	2.13 ± 0.25 $b^{\#}$	2.84 ± 0.39 c*	1.81 ± 0.25 d^{NS}

Values are expressed as mean ± SD of six rats each group.
Units: Protein carbonyls- nmoles/mg proteins. 'a' as compared with group I; 'b' as compared with group II; 'c' as compared with group II; 'd' as compared with group I; statistical significance - #p < 0.01, *p < 0.05; NS-not significant.

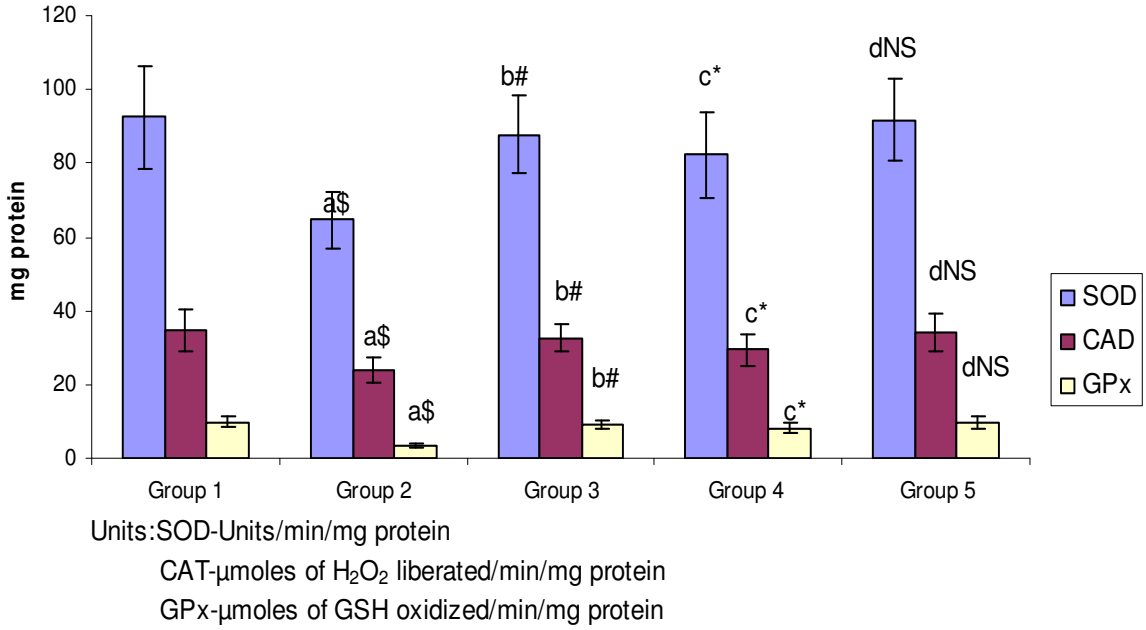

Figure 2. The levels of antioxidant status in stomach of control and experimental animals (mean ± SD, N = 6). Values are expressed as mean ± SD of six rats each group. a-compared with group I; b-compared with group II; c-compared with induced group II; d-compared with group I; statistical significance - $^{\$}p < 0.001$, $^{\#}p < 0.01$, *p < 0.05; NS-not significant.

compared with control rats.

DISCUSSION

In gastric carcinoma bearing rats, there was a sharp drop in their body weight. Weight loss is one of the most frequent adverse systemic effects of malignancy (Dewys et al., 1980). A decline in food intake relative to energy expenditure is the fundamental physiological derangements leads to cancer associated weight loss (Mulligan and Tisdale, 1991; Pain et al., 1984). During gossypol treatment showed gradual increase in body weight. This indicates the antineoplastic property of the drug. Nutritional therapy is a key component for the treatment of cancer cachexia and to actually help in controlling malignant disease in some situations (Ogilvie and Vail, 1990).

Lipid peroxidation is an important cause of cell membrane damage since it has been shown that lipid peroxidation degrades the poly unsaturated fatty acid of cell membrane with consequent disruption of membrane integrity (Niki, 1987; Fridorich, 1986). Lipids are modified by ROS and visualized as a thiobarbituric acid reactive substance (TBARS). Oxidative damage to proteins generates increased carbonyl groups due to oxidation of sensitive amino acids, such as histidine, proline, arginine and lysine (Young et al., 2007). We measured the TBARS and protein carbonyls which serve as an indicator for intracellular oxidation in gastric mucosa. The increase in

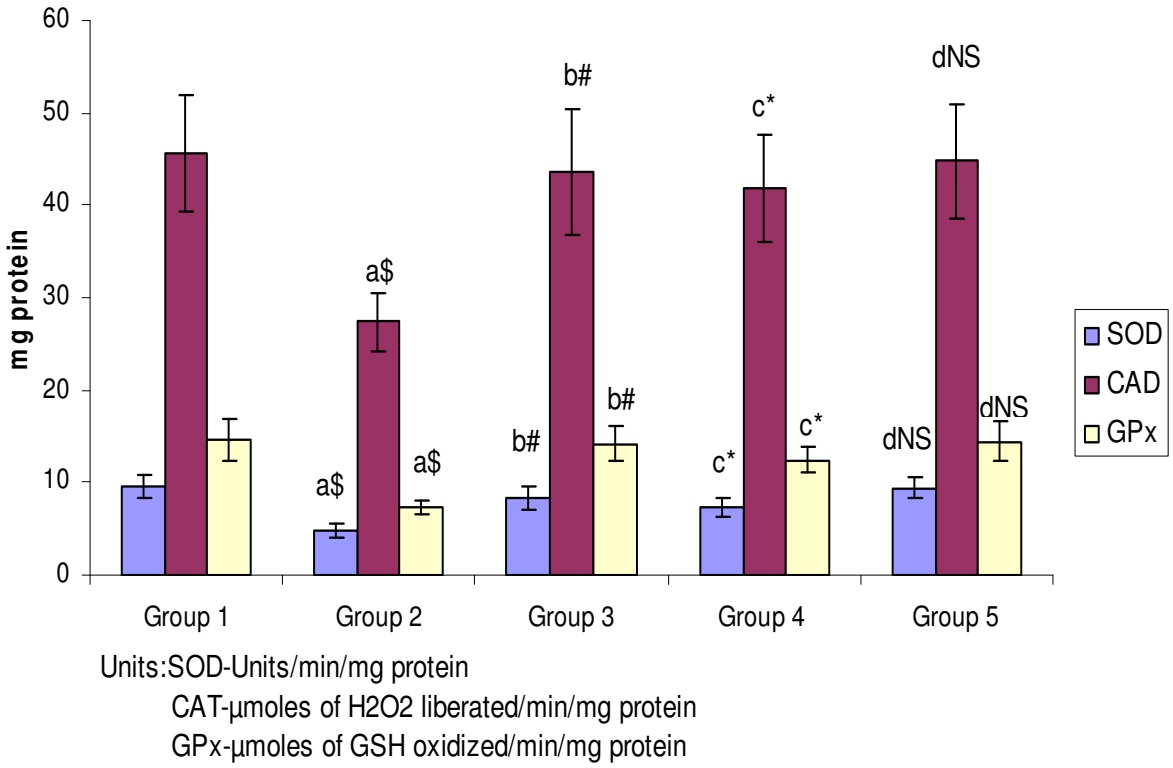

Figure 3. The levels of antioxidant status in liver of control and experimental animals.
(mean ± SD, N = 6). Values are expressed as Mean ± SD of six rats each group. a-compared with group I; b-compared with group II; c-compared with induced group II; d-compared with group I; statistical significance - $^{\$}p<0.001$, $^{\#}p<0.01$, $^{*}p<0.05$; NS-not significant.

Table 3. The levels of GSH, GR and G6PDH in stomach of control and experimental animals (mean ± SD, N = 6).

Treatment groups	Group I: Control alone	Group II: MNNG treated	Group III: Gossypol pretreated	Group IV: Gossypol post treated	Group V: Gossypol alone
GSH	12.59 ± 2.10	06.58 ± 2.00 $a^{\#}$	11.79 ± 1.67 $b^{\#}$	09.84 ± 1.50 c*	12.19 ± 1.96 d^{NS}
GR	32.78 ± 4.30	18.44 ± 2.50 $a^{\$}$	27.88 ± 3.60 $b^{\#}$	24.51 ± 3.20 c*	31.38 ± 4.10 d^{NS}
G6PDH	4.17 ± 0.50	12.45 ± 1.50 a^{*}	5.85 ± 0.70 $b^{\$}$	8.23 ± 0.90 $c^{\#}$	4.07 ± 1.10 d^{NS}

Values are expressed as mean ± SD of six rats each group. a-compared with group I; b-compared with group II; c-compared with induced group II; d-compared with group I; statistical significance - $^{\$}p < 0.001$, $^{\#}p < 0.01$, $^{*}p < 0.05$; NS-not significant.

Table 4. The levels of GSH, GR and G6PDH in liver of control and experimental animals (mean ± SD, N = 6).

Treatment groups	Group I: Control alone	Group II: MNNG treated	Group III: Gossypol pretreated	Group IV: Gossypol post treated	Group V: Gossypol alone
GSH	4.19 ± 0.50	1.89 ± 0.20 $a^{\$}$	3.92 ± 0.50 b*	3.39 ± 0.60 c*	4.08 ± 0.60 d^{NS}
GR	34.51± 5.30	15.65 ± 3.60 $a^{\$}$	29.47 ± 3.80 $b^{\$}$	28.09 ± 4.80 $c^{\$}$	32.36 ± 4.30 d^{NS}
G6PDH	9.28 ± 1.30	3.86 ± 0.40 $a^{\$}$	7.78 ± 1.10 b^{NS}	6.28 ± 0.80 $c^{\#}$	9.31 ± 0.90 d^{NS}

Values are expressed as mean ± SD of six rats each group. a-compared with group I; b-compared with group II; c-compared with induced group II; d-compared with group I; statistical significance - $^{\$}p < 0.001$, $^{\#}p < 0.01$, $^{*}p < 0.05$; NS-not significant.

lipid peroxide may suggest a possible mechanism of tissue injury by reactive oxygen intermediates (Bonnes et al., 1992). This may lead to permanent alterations in the genetic material which may serve as an initial step in the

process of carcinogenesis. In recent years, there is convincing evidence that free radicals can stimulate cancer development at all the three stages of cancer development directly through lipid peroxidation (Dreher, 1996). MNNG is a very effective carcinogen in interacting with membrane lipids and consequently inducing free radical formation (Velmurugan and Nagini, 2005). Hence exposure of MNNG to an acidic environment such as that prevailing in the stomach was shown to generate free radicals (Nagara et al., 1972). In addition, the reaction of MNNG with hydrogen peroxide was demonstrated to produce highly toxic hydroxyl radicals capable of causing deleterious effects at sites away from the target tissue (Mikuni et al., 1985). In group 2, animals due to action of MNNG on stomach and liver, the hydroxyl radicals are generated which leads to oxidation of structural and functional proteins, membrane lipids and depletion of glutathione. Lipid peroxidation causes loss of membrane fluidity, impairs ions transport and finally leads to loss of cellular functions. The gossypol treated groups reverts the cellular function, inhibits the hydroxyl radical formation and increases the concentration of antioxidants.

The antioxidants play an important role in preventing the cells from oxidative damage. In our present study, the activities of antioxidant enzymes such as SOD, CAT, Gpx, GSH, GR and G6PDH were found to be decreased significantly in gastric cancer bearing animals. Antioxidant enzymes scavenge intermediates of oxygen reduction process and provide the primary defense against cytotoxic oxygen radical. Superoxide dismutase is the only enzyme that disrupts superoxide radical and is present in all cells with high amount in erythrocytes (Beulter and Gelbert, 1983). Decreased SOD activity had been reported in various cancerous conditions (Vand Driel et al., 1997; Selvendiran et al., 2003). Our present study is also showing decreased activity of SOD in cancer bearing animals. Catalase is widely distributed in all tissues and more in liver. It catalyses the breakdown of hydrogen peroxide from cells as it decomposes the hydrogen peroxide to oxygen and water. Several reports have cited decreased activities of SOD and catalase in various carcinogenic conditions (Floyd, 1982; Thirunavukarasu and Sakthisekaran, 2001), parallel with this observation in the present study we have observed a decline in SOD and CAT activities in cancer bearing animals, which may be due to the increase in circulating lipid peroxides. Decrease in SOD activity results in accumulation of superoxide anions, a highly diffusible and potent oxidizing radical capable of traversing membranes and causing deleterious effects even in sites far from the tumor (Oberlay and Buettner, 1979). The decreased activities of CAT found in the cancerous conditions may be due to the exhaustion of these enzymes in catalyzing the over production of hydrogen peroxides by the cancerous cells. Moreover, CAT possess a slow catalyst activity at low intracellular levels of its substrate hydrogen peroxides and under this condition, Gpx also plays a predominant role in the detoxification of peroxides from the cell or tissues (Thirunavukarasu et al., 2001). Gpx activity was decreased in cancer bearing animals. This may be due to the accumulation of free radical, thereby impaired antioxidant system occur in cancer bearing animals (Guven et al., 1999), whereas in gossypol treated animals, the level of Gpx activity is increased when compared with cancer bearing animals. Upon gossypol treatment, it suggests that it could have protected the cell/tissues against the cytotoxic effects of carcinogen and it may reduce the proliferation of cancerous cell.

Glutathione is a tripeptide of glycine, glutamic acid and cysteine. In the red blood cell, the reduced form of glutathione is vital in maintaining hemoglobin in a reduced state and hence protecting the cells from oxidative damage. Glutathione is involved in detoxification of hydrogen peroxide through glutathione oxidase. GSH is an important non-protein thiol and in conjunction with Gpx and GST, plays an important role in protecting cells against cytotoxic and carcinogenic chemicals by scavenging reactive oxygen species. We have observed that in cancer bearing animals, the GSH levels are significantly decreased. This indicates that GSH pathway is susceptible to oxidation in cancer bearing animals, whereas in gossypol treated animals, the GSH levels are significantly increased. This shows that gossypol could protect cells against cytotoxic effect and stimulate the antioxidant enzymes. The liver is the main organ with the highest content of GSH and supplies GSH to extra hepatic tissues. It plays a major role in the inter-organ homeostasis of glutathione (Punekar, 1991). Glutathione efflux occurs as reduced glutathione which is mainly exported into circulation (Fernandez Checa, 1992). Depletion of GSH and decreased activity of GR in the stomach and liver probably serves to maintain high levels of GSH in the tumors. The decrease in levels of GSH in stomach and liver observed in our cancer bearing animals may be due to the increased utilization of GSH by glutathione-S-transferase (GST) in detoxification of endogenously or exogenously exposed carcinogens (Ghalia et al., 2000). Increase in NADPH production rate depends on glucose availability and the function of the redox sensitive glucose-6-phosphate dehydrogenase which is the rate limiting enzyme in the pentose phosphate pathway. Decrease in the activity of G6PDH was observed in the MNNG treated rats in liver and stomach. It shows that G6PDH activity may be utilized by the cancer cells in stomach. It was discovered early in the 1920s that cancer cell constitutively up regulate glucose metabolism (Warburg, 1930). Thus, cancer cells tend to synthesis ATP mainly through ‘glycolysis’, a metabolic state that is linked to high glucose uptake and local acidification owing to lactate production. Since gossypol has cytotoxic activity it may kill the cancerous cells and the utilization of G6PDH levels were reverted back to normal range so, activation of the pentose phosphate

pathway maintains NADPH level in gossypol treated rats.

Hence our present suggests that gossypol inhibits proliferation of cancer cells and protects the cells from cytotoxic damage induced by free radicals by acting the antioxidant enzymes. From the review of literature it is clear that gossypol has been reported to have potent anticancer activities in many types of cancer (Balci et al., 1999; Band et al., 1989; Wang et al., 2000). Our results suggest that a gossypol treatment offers a promising effect by acting as a potential chemotherapeutic chemopreventive drug against gastric carcinogenesis.

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