1α, 25-dihydroxyvitamin D₃ prevents DNA damage and restores antioxidant enzymes in rat hepatocarcinogenesis induced by diethylnitrosamine and promoted by phenobarbital

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INTRODUCTION

To investigate the chemopreventive effects of 1α, 25-dihydroxyvitamin D₃, in diethylnitrosamine induced, phenobarbital promoted rat hepatocarcinogenesis. A number of micronutrients, macronutrients and non-nutrients have been reported as the chemopreventive agents in the carcinogenesis[2]. Vitamin D₃, treatment of mice with GM-CSF-secreting tumors can interrupt the myelopoesis-associated immunosuppressor cascade and reduce tumor metastasis[3]. Various new vitamin D analogues are developed with increased growth inhibitory and reduced calcemic activity, but significant antiproliferative and differentiation-inducing agents have now been synthesized and may be used as anticancer drugs[4]. Polychlorinated biphenyls, phenobarbital and many other compounds that induce hepatic biotransformation enzymes promote experimental hepatocarcinogenesis in rodents previously exposed to initiating carcinogens[5]. Several mechanisms for liver tumor promotion by PB and other inducing xenobiotics have been documented[6].

Number of methods are available for detecting DNA damage, as opposed to the biological effects of DNA damaging agents (e.g., micronuclei, mutations, structural chromosomal aberrations) have been used to identify substances with genotoxic activity. The alkaline elution assay ignores the critical importance of intercellular differences in DNA damage and requires relatively large numbers of cells. The full approach for assessing DNA damage is the single-cell gel electrophoresis (SCG) or comet assay[7]. Identification of different cell populations can be made by a modified alkaline comet assay[8]. Comet assay can be used to identify possible human mutagens and carcinogens[9] and DNA damage of human hepatoma cells irradiated by heavy ions[10]. The alkaline comet assay has been very popular for the analysis of DNA damage caused by various chemical and physical agents[11,20]. The genetic damages in leprosy patients undergoing multidrug treatment are also measured by comet assay[21].

Free radical species are involved in carcinogenesis, superoxide dismutases catalyze the dismutation of super-oxide

CONCLUSION: Supplementation of 1α, 25-(OH)₂D₃ has a marked protection against hepatic nodulogenesis, antioxidant enzymes and DNA damages in DEN induced rat hepatocarcinogenesis promoted by phenobarbital.

LIVER CANCER
radical to hydrogen peroxide and oxygen. Chemical induction of liver carcinoma is associated with changes in the oxygen radical metabolism in liver. The changes in hepatic oxygen radical metabolism were demonstrated by measurement of the antioxidant enzymes SOD. Tumour cells have abnormal activities of antioxidant enzymes, and decreased activities of SOD in tumour cells. The influence of oxygen-derived free radicals on survival in advanced colonic cancer was assessed in a prospective randomized controlled double-blind trial using the radical scavengers. Compounds that can scavenge excessive free radicals in the body are suggested to hinder the process of carcinogenesis.

The present study was undertaken to investigate the effectiveness of 1α, 25(OH)₂D₃ on the development of hepatic nodules, the cytogenetic effects of DEN induced rat hepatocarcinogenesis determined by comet assay and the antioxidant enzymes in diethylnitrosamine induced rat hepatocarcinogenesis promoted by phenobarbital.

**MATERIALS AND METHODS**

**Chemicals**

All the reagents and biochemicals, unless otherwise mentioned, were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

**Animals**

Male Sprague-Dawley rats (80-100 g) were purchased from the Indian Institute of Chemical Biology (CSIR), Kolkata, India. They were given the standard laboratory diet purchased from Lipton, India. The animals were housed in an air-conditioned room (22±1°C, relative humidity 50±10%) with a 12-h light/dark cycle in Tarson cages (4 rats per cage) and were acclimatized for 1 wk before the start of the experiment. Guidelines for the care and use of the laboratory animals (National Institute of Health, USA) were followed during the experiment and approved by the Institutional Animal Ethics Committee (IAEC), Jadavpur University, Kolkata.

**Experimental regime**

The rats were randomly divided into 6 different groups with 10 rats in each as illustrated in Figure 1. Groups A, C and E rats received a single intraperitoneal (i.p) injection of DEN (Sigma) at a dose of 200 mg/kg body mass in 9 g/L NaCl solution at 4 wk of age while group B served as normal vehicle control (received normal saline once). After a brief recovery of 2 wk, all the DEN treated rats were given PB at 0.05% daily in the basal diet till wk 20. Group A was DEN control. Treatment of 1α, 25(OH)₂D₃ in group C was started 4 wk prior to DEN injection and continued thereafter till wk 20 at a dose of 0.3 µg/100 µL propylene glycol per os (opus sit) twice a week. In group E 1α, 25(OH)₂D₃ treatment at the same dose mentioned as above was started 1 wk after DEN injection and continued thereafter till the completion of the experiment. The animals of groups D and F served as 1α, 25(OH)₂D₃ controls for groups C and E that received 1α, 25(OH)₂D₃ (Sigma, MO, USA) at a dose of 0.3 µg/100 µL propylene glycol per os twice weekly for 20 wk. All the treatments were withdrawn at wk 20 and the rats were sacrificed at wk 21 under proper ether anaesthesia.

**Comet assay**

Comet assay was performed on liver tissue under alkaline conditions following the procedure of Ward et al., with minor modifications. All the steps of comet assay were conducted under yellow light to prevent the occurrence of additional DNA damage. After sacrifice, liver of either lobe was excised, minced and homogenized in 50 µL of phosphate-buffered saline (PBS; pH 7.5). Briefly, 4 µL of homogenized tissue samples was diluted with 50 µL of PBS and mixed with 150 µL of 10 g/L low melting point agarose (37°C) prepared in PBS and pipetted onto an 10 g/L normal melting agarose precoated slide, which had been dried overnight, and covered with a coverslip. After the slide was kept on a chilled plate for 10 min, the coverslip was removed and the slide was lowered into freshly made ice-cold lysis solution (2.5 mol/L NaCl, 100 mmol/L EDTA, 10 mmol/L Tris, 100 g/L DMSO, 10 g/L Triton X-100, pH 10) and kept at 4°C in the dark for 60 min. After draining the lysis solution, the slide was rinsed with distilled water for 15 min. After washed twice in the prepared destained water, the slide was placed in a horizontal electrophoresis tank containing freshly made buffer (300 mmol/L NaOH and 1 mmol/L EDTA, pH >13) for 30 min. Electrophoresis was performed in the same buffer for 20 min by applying an electric field of 25 V (0.8 V/cm) and adjusting the current to 300 mA by slowly changing the buffer level in the tray. After electrophoresis the slide was rinsed gently with 0.4 mol/L Tris-Hcl buffer (pH 7.5) for 5 min, this step was again repeated. Then the slide was dried at room temperature and kept in a refrigerator in a sealed container until analysis. Duplicate slides were prepared for all the samples.

The slides were immersed in distilled water for 30 min, then stained with 100 µL of ethidium bromide (5 µg/mL) and read at 250x under a Zeiss fluorescence microscope equipped with a green excitation filter and a 590 nm barrier filter. All slides were coded and examined blindly. Routinely 100 cells (50 cells/slide) were screened per sample. In selecting cells for measurements, straight line scanning of a slide was begun at an arbitrary point and cells were measured as they came into the field, provided there was no overlap with patterns from other cells. The length and width of the DNA mass were measured using an ocular micrometer disk. Under these conditions a DNA pattern with a ratio of one had a DNA length of ~25 µm and a ratio of four had a DNA length of ~100 µm. The length: width ratios of the DNA mass and the frequency of tailed (damaged) cells were used in all comparisons.

**Figure 1 Basic experimental protocol.**

**Morphology and morphometry of liver tissue**

After the rats were sacrificed, their livers were excised from all
treated and control rats, weighed and examined on the surface for subcapsular macroscopic lesions (hyperplastic nodules; HNs). The nodules with approximate spheres were measured in 2 perpendicular directions to the nearest millimeter into three categories namely \( \geq 3, <3 \rightarrow 1 \) and \( \leq 1 \) mm\(^2\). [27].

### Histopathological examination
For histopathological examination and morphometric analysis, tissues were fixed in 40 g/L buffered formaldehyde and the fixed paraffin embedded sections were stained with hematoxylin and eosin (H&E).

### Histochemical detection of γ-glutamyl transpeptidase-positive foci
After sacrifice of the rats, each liver was examined of the right, left and caudate lobes. They were fixed in an ice-cold mixture of dehydrated ethanol and glacial acetic acid (19:1) for 4 h followed by an overnight incubation in 995 mL/L ethanol at 4 °C and then embedded in soft paraffin (melting point 47 °C). Two contiguous paraffin sections were made from each liver section for γ-glutamyl transpeptidase (GTT) histochemistry according to the method of Rutenberg [30]. Quantitative evaluation of GTT-positive foci (lesions smaller than a liver lobule mainly visible microscopically) was performed according to the method of Campbell [30].

### Biochemical assays
The animals were sacrificed with proper anaesthesia. Liver of either lobes was minced and homogenized with 0.25 mol/L sucrose and the homogenate was centrifuged at 9 000 g (33 000 r/min) for 15 min in refrigerated centrifuge (Megaflue 1.0R). The pellet was discarded and an aliquot of supernatant was kept for the assay of cytosolic enzyme activities. The left portion was centrifuged at 105 000 g for 90 min in refrigerated centrifuge (Megaflue 1.0R). The microsomal fraction was prepared separately from hyperplastic nodule (HNs) and non-nodular surrounding parenchyma (NNSP) liver area and untreated normal control liver. All the operations were done at 0-4 °C.

The activity of superoxide dismutase (SOD) was measured by following the method of Beyer and Fridovich [32]. Hepatic cytosolic enzyme activities were determined with 1-chloro 2,4-dinitrobenzene as the substrate according to the method of Habig et al. [33].

### Statistical analysis
Data were analyzed statistically for differences between the means using Dunnett’’s t-test when more than one group was compared against a control group. P-value <0.05 was considered statistically significant.

## RESULTS
### Dietary intake
Daily food and water intake of all the groups of rats was same. Daily intake of water was measured with a measuring cylinder and it was found that each rat took on an average of 8-10 mL water per day.

### Body and liver mass
The effect of 1α, 25(OH)\(_2\)D\(_3\) on body and liver mass of different group of rats sacrificed after 20 wk is shown in Table 1. Body mass of DEN control group rats (group A) were slightly lower (\( P<0.05 \)) than that of the normal control rats (group B). Treatment with 1α, 25(OH)\(_2\)D\(_3\) increased the final body mass of the animals in groups C, D, E and F which received 1α, 25(OH)\(_2\)D\(_3\) as compared with group A carcinogen control. This suggested that treatment with 1α, 25(OH)\(_2\)D\(_3\) had no adverse effect on the growth response of rats. Liver masses of rats in various groups showed no significant differences. The relative liver mass of DEN control group rats (group A) was found to be significantly increased (\( P<0.01 \)) than that of normal control rats (group B).

### Effect of 1α, 25(OH)\(_2\)D\(_3\) on induction of GTT-positive foci
Table 2 shows that GTT-positive foci developed in all the DEN treated groups (Groups A, C and E), while the livers of rats in normal as well as 1α, 25(OH)\(_2\)D\(_3\) control groups (groups B, D and F respectively) were found to be normal in terms of histochemical observations of GTT-positive foci. In group E supplementation of 1α, 25(OH)\(_2\)D\(_3\) inhibited the appearance of GTT-positive foci (45.25%). But 1α, 25(OH)\(_2\)D\(_3\) treatment which was started 4 wk before DEN administration and continued till the end of the experiment minimized the appearance of GTT-positive foci most significantly in group C (68.80%) than in DEN treatment. Thus 1α, 25(OH)\(_2\)D\(_3\) decreased significantly (\( P<0.01 \)) GTT-positive foci in group C compared to the DEN control (group A).

### Effect of 1α, 25(OH)\(_2\)D\(_3\) on hepatic histopathology
Histopathological examination of liver sections from normal untreated group B (Figure 5) revealed normal liver parenchymal cells with granulated cytoplasm and small uniform nuclei radially arranged around the central vein. In the DEN treated groups A, C and E, phenotypically altered hepatocytes in altered liver cell foci and nodules at varying extent were noticeable throughout the hepatic parenchyma. The hepatocellular architecture of DEN control (group A) was found to be grossly altered and the hepatocytes became oval and small uniform nuclei radially arranged around the central vein.

### Table 1 Body and liver masses in each group of rats at end of the study (after 20 wk) (mean±SE)

| Group | No. of rats | Body mass (g) | Liver mass (g) | Relative liver mass (g) (Liver/100 g body mass) |
|-------|-------------|---------------|---------------|-----------------------------------------------|
| A     | 6           | 282.5±15.8    | 14.6±2.6      | 5.16±0.47                                     |
| B     | 10          | 338.0±18.3    | 10.8±1.9      | 3.19±0.21                                     |
| C     | 8           | 334.3±13.8    | 12.3±1.8      | 3.67±0.36                                     |
| D     | 10          | 348.9±21.6    | 11.9±1.5      | 3.41±0.29                                     |
| E     | 8           | 308.1±39.1    | 13.1±2.2      | 4.25±0.45                                     |
| F     | 10          | 336.6±22.2    | 12.1±1.8      | 3.59±0.42                                     |

Table 2 Effect of 1α, 25(OH)\(_2\)D\(_3\) on inhibition of GTT-positive foci in DEN induced rat hepatocarcinogenesis promoted by phenobarbital (mean±SE)

| Group | No. of rats | No. of GTT-positive foci/cm² | Decrease (%) |
|-------|-------------|-----------------------------|--------------|
| A     | 06          | 26.16±2.16                  |              |
| C     | 08          | 8.16±0.59                   | 68.80        |
| E     | 08          | 14.32±1.23                  | 45.25        |

Table 3 Amount of positive foci in DEN treated rats (mean±SE)
in shape. The altered liver cells in foci and nodules were considerably enlarged, vesiculated and binucleated (Figure 4). A substantial irregularity (enlargement) in the shape of nuclei and chromatin pattern (chromatin condensation) were also observed. The nucleo-cytoplasmic ratio was increased in sinuses and greatly dilated with hyperplastic Kupffer cells. The cytoplasm was extensively vacuolated, continued masses of acidophilic material were observed. Supplementation of $\alpha$, $25(OH)\alpha_2$, $25(OH)\beta_3$ for the entire period study (group C) elicited a maximum protection against DEN induced hepatocarcinogenesis, which was reflected in almost normal hepatocellular architecture. In group C liver cells were found to contain compact cytoplasmic material with only clear cell foci (Figure 6). The nucleo-cytoplasmic ratio was decreased considerably as compared to group A. A considerable vacuolation was still observed in the cytoplasm and the compactness of hepatocytes was somewhat disturbed in group C. The liver sections from these groups presented a predominance of clear cell foci rather than eosinophilic or basophilic foci. There was a slight decrease in nucleo-cytoplasmic ratio in the cells with respect to group A with slightly dilated sinuses. The number of binucleated cells was less as compared to group A with normal size nuclei.

### Effect of $\alpha$, $25(OH)\alpha_2$, $25(OH)\beta_3$ on incidence, number and size of hepatocellular lesions

The carcinogen treated rats showed 100% nodule incidence in group A as compared to the normal control rats (group B). The incidence of HNs was lower in the rats that received $\alpha$, $25(OH)\alpha_2$, $25(OH)\beta_3$ and DEN in groups C and E (Table 3). The average number of nodules/nodule-bearing livers (nodule multiplicity) was lower as compared to group A. The result was statistically significant ($\alpha$, $25(OH)\alpha_2$, $25(OH)\beta_3$) on incidence, number and size of HNs as compared to group A. The total number of nodule 22 and the average nodule bearing livers (3.66±0.68) in group C were compared with the DEN control (group A). Group C rats showed the lowest value in each range compared with the other groups (A and E). In the present study the relative size distribution of nodules revealed that supplementation of $\alpha$, $25(OH)\alpha_2$, $25(OH)\beta_3$, characteristically reduced the appearance of HNs more than 3 mm in size in group C compared to group A.

### Effect of $\alpha$, $25(OH)\alpha_2$, $25(OH)\beta_3$ on comet Assay

The results of comet assay based on mean tailed cells (TC) (%) and mean length: width ratios (L: W) in hepatic cells of rats treated with DEN are shown in Table 4. The mean length to width ratio of the DNA mass observed in DEN control rats (group A) was significantly greater ($\alpha$, $25(OH)\alpha_2$, $25(OH)\beta_3$, compared with the normal control (group B). A significant decrease ($\alpha$, $25(OH)\alpha_2$, $25(OH)\beta_3$) in DNA damage on Comet Assay was observed in both HNs and NNNSP tissues in group C rats, where $\alpha$, $25(OH)\alpha_2$, $25(OH)\beta_3$ treatment started 4 wk before DEN administration and continued till the end of the experiment. In group E, $\alpha$, $25(OH)\alpha_2$, $25(OH)\beta_3$, supplementation only for 15 wk, starting the treatment 1 wk after DEN administration showed no statistical significance in SOD activity.

### Table 3 Effect of $\alpha$, $25(OH)\alpha_2$, $25(OH)\beta_3$ on incidence, number and size of hepatocellular lesions during DEN induced hepatocarcinogenesis promoted by phenobarbital (mean±SE)

| Group | No. of rats with nodules | Nodule incidence (%) | Total No. of nodule | Average No. of nodules per nodule bearing liver | Relative size |
|-------|--------------------------|----------------------|---------------------|-----------------------------------------------|--------------|
| A     | 10/10                    | 100                  | 168                 | 16.80±0.33                                    | <1 mm        |
| C     | 6/10                     | 60%                  | 22                  | 3.66±0.88                                     | >1 mm<3 mm   |
| E     | 8/10                     | 80%                  | 72                  | 8.00±0.05                                     | >3 mm        |

Statistical level of significance by using Dunnett’s t-test, $\alpha$ <0.01, compared with control. $\alpha$ <0.05 compared with group A.
Effect of 1α, 25(OH)₂D₃ on hepatic cytosolic lipid peroxidation

1α, 25(OH)₂D₃ had effect on hepatic cytosolic lipid peroxidation (Table 6) in different groups of rats treated with DEN, which was promoted by phenobarbital. A significant increase in the total content of MDA (P<0.01) was observed in DEN control rats (group A), both HNs and NNSP liver area in group A were compared with normal control. A significant decrease (P<0.05) in elevated hepatic MDA level was found in group C when compared with DEN control. Supplementation of 1α, 25(OH)₂D₃ led to a significant reduction in total MDA production in DEN treated rats. The maximum effect was observed in group C rats whose 1α, 25(OH)₂D₃ treatment was started 4 wk before DEN administration and continued till 20 wk, which offered a better protection in group E, in which 1α, 25(OH)₂D₃ was supplemented for only 15 wk, starting 1 wk after DEN administration.

Table 6 Changes in total hepatic lipid peroxidation (nmol MDA/100 mg protein) level in different groups of rats treated with 1α, 25(OH)₂D₃ during DEN induced rat hepatocarcinogenesis promoted by phenobarbital (mean±SE, n=5)

| Groups | Nodules       | Surrounding | Control      |
|--------|---------------|-------------|--------------|
| A      | 18.72±1.59    | 17.4±1.34   | 2.44±0.84    |
| C      | 8.80±0.02     | 7.6±0.88    | 3.16±0.94    |
| E      | 13.16±1.23    | 12.32±1.03  | 2.8±0.79     |

Statistical level of significance by using Dunnett’s t-test. *P<0.01, †P<0.05 compared with control; ‡P<0.05 compared with group A.

Effect of 1α, 25(OH)₂D₃ on hepatic cytosolic glutathione (GSH)

Table 7 shows the GSH content in different experimental groups. GSH content was found to be increased both in HNs and NNSP liver areas (P<0.01) in DEN control rats (group A) compared with normal control (group B). GSH content was decreased significantly (P<0.05) in group C. GSH levels were marginally increased in groups D and F when compared with the normal control.

Table 7 Changes in total hepatic reduced glutathione (GSH) (mg/100 g tissue) level in different groups of rats treated with 1α, 25(OH)₂D₃ during DEN induced rat hepatocarcinogenesis promoted by phenobarbital (mean±SE, n=5)

| Groups | Nodules       | Surrounding | Control      |
|--------|---------------|-------------|--------------|
| A      | 367.6±25.5    | 310.0±24.9  | 226.0±22.5   |
| C      | 268.0±21.5    | 254.0±20.1  | 248.0±21.7   |
| E      | 303.2±22.5    | 284.0±21.6  | 256.0±22.5   |

Statistical level of significance by using Dunnett’s t-test. *P<0.01, †P<0.05 compared with control; ‡P<0.05 compared with group A.
Effect of 1α, 25(OH)2D3 on 1-chloro-2, 4-dinitro benzene (CDNB) conjugated hepatic cytosolic glutathione S-transferase (GST) activity in different groups

Table 8 depicts the GST activity with CDNB in different experimental groups. DEN control rats showed (group A) a significantly increase (P<0.01) more than 2-fold in NNSP compared with the normal control, but HNS showed a significantly decrease in value. Altered activity of 1α, 25(OH)2D3 was found in group B. Groups C and E also showed higher altered activity of 1α, 25(OH)2D3 than the normal control rats. 1α, 25(OH)2D3 was found to be more effective in the inhibition of rat liver carcinogenesis (P<0.05) in abating the GST activity. 1α, 25(OH)2D3 control groups (groups D and F) had no statistical significance in the GST activity when compared with normal control.

Table 8 Changes in activity of 1-chloro-2, 4-dinitro benzene (CDNB) conjugated (μmol CDNB conjugated/ mg protein/mL) hepatic cytosolic glutathione S-transferase (GST) in different groups of rats treated with 1α, 25(OH)2D3 during DEN induced rat hepatocarcinogenesis promoted by phenobarbital (mean±SE, n=5)

| Groups | Nodules | Surrounding | Control |
|--------|---------|-------------|---------|
| A      | 2.52±0.23| 1.54±0.23   | 0.9±0.10 |
| C      | 1.45±0.27| 1.24±0.11   | 1.12±0.13 |
| E      | 1.76±0.29| 1.48±0.19   | 1.10±0.11 |

Statistical level of significance by using Dunnett’s t-test. *P <0.01, **P <0.05 compared with control. *P <0.05 compared with group A.

DISCUSSION

The results of our present investigation clearly demonstrated that long term supplementation of 1α, 25(OH)2D3 (at a dose of 0.3 μg/100 μL propylene glycol twice a week) greatly reduced the incidence of hepatic nodulogenesis, antioxidant enzymes and genetic damage in DEN induced rat hepatocarcinogenesis promoted by phenobarbital. Previous studies in our laboratory have shown that long term supplementation of 1α, 25(OH)2D3, in combination with vanadium could effectively inhibit DEN-induced rat liver carcinogenesis[34,38].

DEN is a well-known hepatocarcinogen in rats, forming DNA-carcinogen adducts in the liver and inducing hepatocellular carcinomas without cirrhosis through the development of putative preneoplastic enzyme-altered hepatocellular focal lesions[37]. After limited treatment with DEN, the rats ended up with a large benign hepatomas[39,40], which were equivalent to neoplastic nodules and highly differentiated hepatocarcinomas. The preneoplastic lesions were thought to be the possible precursors of hepatic cancer in experimental animals and humans[39]. Treatment with hepatocarcinogen could result in the proliferation of oval cells. These cells have been shown to have the ability to differentiate into hepatocytes[40]. The results of our present investigation clearly indicated that long term supplementation of 1α, 25(OH)2D3 could reduce the incidence, multiplicity and size of visible HNs more than 3 mm in size. Prenoeplasia (γ-glutamyl transpeptidase- positive and glucose 6-phosphate negative) appeared in 1α, 25(OH)2D3 treated animals 1 wk after carcinogen withdrawal, but livers from 1α, 25(OH)2D3 depleted rats exhibited an increase in the number of GGT-positive foci. There was no change in the body masses among the groups under study. This is particularly important because nutritional deprivation causing body mass loss might result in a decrease in tumor volume[41]. Thus 1α, 25(OH)2D3 had the maximum effect in reducing the number and nodule growth, which were not mediated through the impairment of nutritional status in the experimental animals. 1α, 25(OH)2D3 effect observed in this study might be important for cancer prevention.

Comet assay is sensitive, a small number of cells and substances are required. It is inexpensive, and easy to apply to any tissues. The tissue selection in our study was based on a recent data analysis of the mice and rats[42]. Comet assay can be applied to any tissues in vivo. In comet assay DNA is organized as loops, retaining the super coils and circular structure that are contained in the nucleosone. Epidemiological studies showed that comet tail was made up of relaxed loops and that the number of loops in the tail could indicate the number of DNA damages and tailed cells consisting of fragments of DNA[43]. This study indicated a significantly higher incidence of DNA damage in the DEN-PB control compared with the normal control. In the present investigation, 1α, 25(OH)2D3 protected against the DNA damage in DEN induced rat hepatocarcinogenesis.

SOD is the first line of defense of the cellular antioxidant system against the oxidative damage mediated by superoxide radicals. Life is continuously exposed to oxidative stress, cells are equipped with gene regulatory mechanism that can sense a high oxidative stress potential and consequently induce higher levels of several enzymes capable of reducing reactive oxygen species and repairing oxidative damage. The antioxidant enzymes are thus a major cell defense against acute oxygen toxicity. Their function is to protect membrane and cytosolic components against damage caused by free radicals. SOD, catalase and glutathione peroxidase (GPx) exemplify some of the most important ones. SOD, catalase could convet oxygen into hydrogen peroxides and water, SOD could diminish the damage caused by superoxide-producing agents as long as the generated hydrogen peroxide was removed by glutathione peroxidase and catalase does not become rate limiting[44,45]. The enzyme GPx and glutathione reductase were then destroyed either by catalase or by enzyme system, these 2 systems could convert hydrogen peroxide into water at the expense of NADPH, using reduced GSH as an electron donor[46]. Differences were found between normal and cancer cell superoxide dismutase activity in the treatment of cancer[47]. The SOD ratio was lower in liver cells, and this might provide an explanation for the higher susceptibility of tumor cells to treatments likely to involve oxygen radicals[48,49]. Our present results showed that 1α, 25(OH)2D3 supplementation in group C inclined towards normal and in group E no change was found. Decreased antioxidant activity could cause the accumulation of free radicals. However, there was no change in SOD activities in group E rats, which might be due to the inactivated gene not reactivated by 1α, 25(OH)2D3 supplementation, the physiological characterization and the genetic mapping of the mutant should identify the gene.

Lipid peroxidation plays an important role in carcinogenesis, treatment with inhibitors of lipid peroxidation, such as vitamins D, E, C, as well as selenium and vanadium and beta-carotene are protective agents[36,39,52]. Stimulation of NADPH-dependent microsomal lipid peroxidation was proposed to be mediated by ADP/Fe2+. NADPH-cytochrome P-450 reductase and cytochrome P-450[53,54]. Therefore, the increase of one of these modulators in the liver could explain the reduced lipid peroxidation. Previous studies in our laboratory showed that a significant increase in the total content of MDA was observed in DEN control compared to normal control[50,51]. In our present study a significant increase in lipid peroxidation was observed in DEN PB-treated rats (group A) when compared with normal control rats (group B). But it was found in group C most significant (P<0.05) compared to DEN-PB control. Treatment with 1α, 25(OH)2D3 abated the production of MDA in different DEN-PB treated rats (groups C and E). The ability of 1α, 25(OH)2D3 to inhibit iron dependent lipid peroxidation in liposomes might be important in protecting the membranes of normal cells against free radical induced oxidative damage[55]. Thus, the oxygen...
radical formation and detoxification, which result in lipid peroxidation and tissue damage, may be prevented.

GSH occurred primarily in the soluble phase, part of it conjugated with foreign compounds or their metabolites for detoxification and transport from body in the precancerous stage. GSH concentration in rat liver increased[56-58]. From our study increased level of GSH in DEN control rats (group A), might be that GGT catalysed the degradation of extracellular GSH that can be used in the intracellular GSH synthesis[59]. 1α, 25(OH)2D3 supplemented groups C and F rats showed decrease in GSH levels. The significantly decreased GSH level in group C reflected the chemopreventive mechanism, by promoting the formation of additional pyridine nucleotides to provide hepatocytes with reduced properties and to inhibit the growth and spread of neoplastic nodules.

GST catalyzes the reaction of compounds with -SH group and thus neutralizes their electrophilic sites and produces more water soluble products. The evolution of thiol dependent detoxification pathways was initially the result of the availability of molecular oxygen[60]. The cellular defence against oxygen free radicals and peroxides is of considerable importance to cell survival. Since the initial report of elevated expression of GST activity in nitrogen mustard resistant cell line[61], a number of different tumors have been found to overexpress GST isoenzymes. In MCF-7 human breast cancer cell line, there appeared to have a coordinately increased expression of both GST-α and other enzymes including selenium dependent glutathione peroxidase and SOD[62]. An increased GST activity was observed in metabolic inactivation and resistance to several anticancer drugs[63].

In the present study, an increased activity of GST was observed in DEN control rats (group A). Decreased activity of GST was observed in groups C and F. The significant decrease (P<0.05) in GST activity was found in group C supplemented with 1α, 25(OH)2D3. The increased anti-cancer drug resistance of one or more of the GST isoenzymes in resistant cells was compared with normal cells[64].

1α, 25(OH)2D3 is very much effective in preventing DEN-PB induced changes in hepatocytes possibly through the inhibition of nodular growth, GGT-positive foci development, normalization of DEN induced changes in enzyme activities and prevention DNA damages in DEN-induced rat hepatocarcinogenesis. The results of our present study strongly suggest that 1α, 25(OH)2D3 may be important for cancer prevention.

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