

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

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Abstract

AIM: To investigate the chemopreventive effects of 1 α , 25-dihydroxyvitamin D₃ in diethylnitrosamine induced, phenobarbital promoted rat hepatocarcinogenesis.

METHODS: The rats were randomly divided into 6 different groups (A-F). Groups A, C and E rats received a single intraperitoneal (i.p) injection of diethylnitrosamine (DEN) 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 phenobarbital (PB) at 0.5 g/L daily in the basal diet till wk 20. Group A was DEN control. Treatment of 1 α , 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 μ g/100 μ L propylene glycol per one single dose (os) twice a week. Group E received the treatment of 1 α , 25-(OH)₂D₃ at the same dose mentioned as above for 15 wk. The rats in group D and F received 1 α , 25-(OH)₂D₃ alone as in group C without DEN injection.

RESULTS: The comet assay showed statistically higher mean values for length to width ratios (L: W) of DNA mass and tailed cells ($P < 0.01$; $P < 0.01$ respectively) in DEN treated rats as compared to their normal controls. Continuous supplementation of 1 α , 25-dihydroxyvitaminD₃ showed a significant ($P < 0.01$) decrease in L:W ratio of DNA mass tailed cells. Furthermore, 1 α , 25-(OH)₂D₃ supplementations elevated the super oxide dismutase (SOD) activity, hepatic malondialdehyde (MDA) level, reduced glutathione (GSH) and glutathione S-transferase (GST) activity ($P < 0.01$, $P < 0.05$, $P < 0.05$ and $P < 0.05$ respectively). As an endpoint marker histological changes were observed to establish the chemopreventive effects of 1 α , 25-dihydroxyvitaminD₃.

CONCLUSION: Supplementations of 1 α , 25-(OH)₂D₃ has

a marked protection against hepatic nodulogenesis, antioxidant enzymes and DNA damages in DEN induced rat hepatocarcinogenesis promoted by phenobarbital.

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INTRODUCTION

1 α , 25dihydroxyvitaminD₃ plays an important role in reducing the incidence of carcinomas of breast, prostate and colon in human as well as in experimental animals^[1,2]. 1 α , 25(OH)₂D₃ has been shown to promote the differentiation of cancer cells and cell lines *in vitro*^[3,4]. Little information is available for the antioxidant property of 1 α , 25(OH)₂D₃ in the inhibition of chemical rat hepatocarcinogenesis^[5]. A number of micronutrients, macronutrients and non-nutrients have been reported as the chemopreventive agents in the carcinogenesis^[6]. Vitamin D₃ treatment of mice with GM-CSF-secreting tumors can interrupt the myelopoiesis-associated immunosuppressor cascade and reduce tumor metastasis^[7]. 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^[8,9]. Polychlorinated biphenyls, phenobarbital and many other compounds that induce hepatic biotransformation enzymes promote experimental hepatocarcinogenesis in rodents previously exposed to initiating carcinogens^[10]. Several mechanisms for liver tumor promotion by PB and other inducing xenobiotics have been documented^[11].

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^[12]. Identification of different cell populations can be made by a modified alkaline comet assay^[13,14]. Comet assay can be used to identify possible human mutagens and carcinogens^[15] and DNA damage of human hepatoma cells irradiated by heavy ions^[16]. The alkaline comet assay has been very popular for the analysis of DNA damage caused by various chemical and physical agents^[17-20]. 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

radical to hydrogen peroxide and oxygen^[22]. 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^[23,24]. 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^[25]. 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 1 α , 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 1 α , 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 μ g/100 μ L propylene glycol *per os* (*opus sit*) twice a week. In group E 1 α , 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 1 α , 25(OH)₂D₃ controls for groups C and E that received 1 α , 25(OH)₂D₃ (Sigma, MO, USA) at a dose of 0.3 μ g/100 μ L 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.*^[26], 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 μ L of phosphate-buffered saline (PBS; pH 7.5). Briefly, 4 μ L of homogenized tissue samples was diluted

with 50 μ L of PBS and mixed with 150 μ L 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 distilled 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 μ L of ethidium bromide (5 μ g/mL) and read at 250 \times using 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 μ m and a ratio of four had a DNA length of -100 μ m. The length: width ratios of the DNA mass and the frequency of tailed (damaged) cells were used in all comparisons.

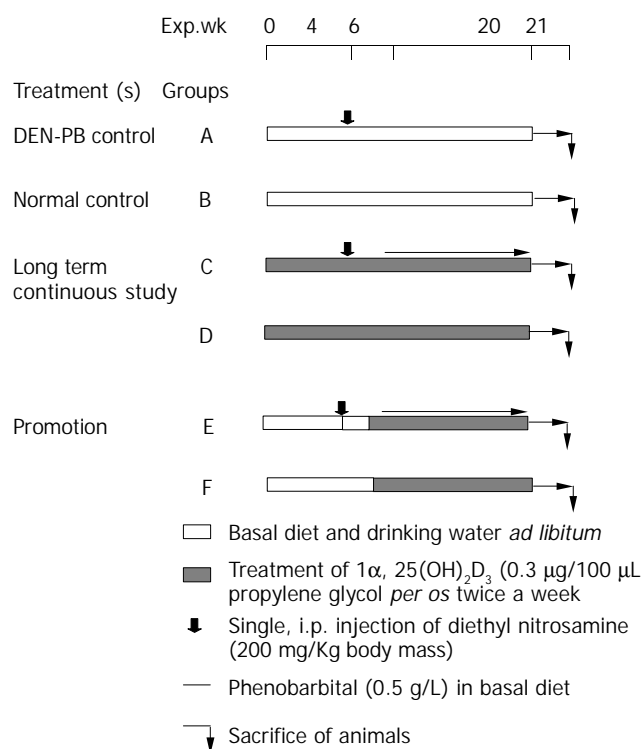


Figure 1 Basic experimental protocol.

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 ≥ 3 , $< 3 - > 1$ and ≤ 1 mm^[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 (GGT) histochemistry according to the method of Rutenberg^[28]. Quantitative evaluation of GGT-positive foci (lesions smaller than a liver lobule mainly visible microscopically) was performed according to the method of Campbell^[29].

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 (Megafuge 1.0R). The pellet was discarded and an aliquot of supernatant was kept for the assay of cytosolic enzyme activities. The left portion was recentrifuge at 105 000 g for 90 min in refrigerated centrifuge (Megafuge 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^[30]. Hepatic cytosolic enzymatic lipid peroxidation was estimated according to the method of Okhaw *et al.*,^[31] based on the formation of malondialdehyde (MDA). The reduced glutathione (GSH) level was quantified by the method of Ellman^[32]. Hepatic cytosolic glutathione S-transferase (GST) activity was 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 $1\alpha, 25(\text{OH})_2\text{D}_3$ 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 $1\alpha, 25(\text{OH})_2\text{D}_3$ increased the final body mass of

the animals in groups C, D, E and F which received $1\alpha, 25(\text{OH})_2\text{D}_3$ as compared with group A carcinogen control. This suggested that treatment with $1\alpha, 25(\text{OH})_2\text{D}_3$ 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). Treatment with $1\alpha, 25(\text{OH})_2\text{D}_3$ significantly ($P < 0.05$) reduced the relative liver masses of rats in groups C, D, E and F compared with group A. $1\alpha, 25(\text{OH})_2\text{D}_3$ supplied rats showed a better resistance against hepatocarcinogenesis.

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

Group	No of rats	Body mass (g)	Liver mass (g)	Relative liver mass (g) (Liver/100 g body mass)
A	6	282.5 \pm 15.8 ^a	14.6 \pm 2.6	5.16 \pm 0.47 ^b
B	10	338.0 \pm 18.3	10.8 \pm 1.9	3.19 \pm 0.21
C	8	334.3 \pm 13.8 ^c	12.3 \pm 1.8	3.67 \pm 0.36 ^e
D	10	348.9 \pm 21.6	11.9 \pm 1.5	3.41 \pm 0.29
E	8	308.1 \pm 19.1	13.1 \pm 2.2	4.25 \pm 0.45
F	10	336.6 \pm 22.2	12.1 \pm 1.8	3.59 \pm 0.42

Statistical level of significance by using Dunnett's *t*-test. ^a $P < 0.05$,

^b $P < 0.01$, significantly different from normal control (group B),

^c $P < 0.05$, ^e $P < 0.05$, significantly different from DEN control (group A).

Effect of $1\alpha, 25(\text{OH})_2\text{D}_3$ on induction of GGT-positive foci

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

Table 2 Effect of $1\alpha, 25(\text{OH})_2\text{D}_3$ on inhibition of GGT-positive foci in DEN induced rat hepatocarcinogenesis promoted by phenobarbital (mean \pm SE)

Group	No. of rats	No. of GGT-positive foci/cm ²	Decrease (%)
A	06	26.16 \pm 2.16	
C	08	8.16 \pm 0.59 ^b	68.80
E	08	14.32 \pm 1.23 ^d	45.25

Statistical level of significance by using Dunnett's *t*-test, ^b $P < 0.01$,

^d $P < 0.01$ compared with group A.

Effect of $1\alpha, 25(\text{OH})_2\text{D}_3$ 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 altered cell foci 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

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 nucleus and chromatin pattern (chromatin condensation) were also observed. The nucleocytoplasmic 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 1 α , 25(OH)₂D₃ 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 nucleocytoplasmic ratio was decreased considerably as compared to group A. The configuration of sinuses appeared normal with normal Kupffer cells. The size of nuclei resembled that of normal cells and binucleated cells were extremely less. A moderate improvement of hepatic histological picture was observed in 1 α , 25(OH)₂D₃ supplemented group C in comparison to group E. 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 decrement in nucleocytoplasmic 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 1 α , 25(OH)₂D₃ 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 1 α , 25(OH)₂D₃ and DEN in groups C and E (Table 3). The average number of nodules/nodule-bearing livers (nodule multiplicity) was also found to be less in 1 α , 25(OH)₂D₃ supplemented group C (60%) as compared to group A. The result was statically significant ($P<0.01$) in group C compared with group A. The total number of nodule 22 and the average nodule bearing livers (3.66 \pm 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 (groups A and E). In the present study the relative size distribution of nodules revealed that supplementation of 1 α , 25(OH)₂D₃ characteristically reduced the appearance of HNs more than 3 mm in size in group C compared to group A.

Effect of 1 α , 25(OH)₂D₃ 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 ($P<0.01$) compared with the normal control group B. Similarly, the mean frequency of tailed cells was (84 \pm 0.020) in DEN control rats and significantly different ($P<0.01$) from the normal control (29 \pm 0.008). Figures 2 and 3 illustrate the distribution of L: W and TC in DEN control

(group A), normal (group B), long term study (group C), and promotion study (group E). For length: width ratio and tailed cells, the median values were higher in DEN control (group A) compared with the normal control (group B). The distribution of damage cells was also wider and the lengths of the boxes were greater in DEN control rats, indicating larger interquartile ranges than that of the normal controls rats. The median line in normal control rats (group B) were tailing towards smaller values. In group C and group E the median lines were in the middle of the boxes, the distribution of TC was more or less symmetrical. In group C 1 α , 25(OH)₂D₃ offered more than 54.09% in the length and width ratio and 53.37% of tailed cells compared to group A.

Table 4 Effect of 1 α , 25dihydroxyvitamin D₃ on DNA damage [based on mean tailed cells (%) and mean length: width ratios \pm SEM of DNA pattern] in hepatic cells of rats during DEN induced rat hepatocarcinogenesis promoted by phenobarbital (mean \pm SE, $n=100$)

Groups	Length and width ratio of DNA mass	Decrease (%)	Tailed cells (%)	Decrease (%)
A	2.44 \pm 0.0681		84 \pm 0.020	
B	1.029 \pm 0.005		29 \pm 0.008	
C	1.12 \pm 0.025 ^b	54.09	39 \pm 0.014 ^d	53.57
D	1.02 \pm 0.0005		29 \pm 0.008	
E	1.63 \pm 0.026 ^g	33.19	62 \pm 0.026 ^f	26.19
F	1.02 \pm 0.005		29 \pm 0.008	

Group A: DEN control; Group B: Normal control; Group C: Long term study; Groups D, F 1 α , 25(OH)₂D₃ control; Group E: promotion. Statistical level of significance by using Dunnett's *t*-test, ^b $P<0.01$, ^g $P<0.01$, ^d $P<0.01$ and ^f $P<0.01$ compared to group A.

Effect of 1 α , 25(OH)₂D₃ on hepatic super oxide dismutase activity

Table 5 depicts the SOD activity. A significant decrease ($P<0.01$) was found in SOD activity in HNs and NNSP tissues in DEN control (group A) compared with the normal control (group B). A significant increase ($P<0.05$) in SOD level was observed in both HNs and NNSP tissues in group C rats, where 1 α , 25(OH)₂D₃ treatment started 4 wk before DEN administration and continued till the end of the experiment. In group E 1 α , 25(OH)₂D₃ was supplemented only for 15 wk, starting the treatment 1 wk after DEN administration showed no statistical significance in SOD activity.

Table 5 Changes in activities of superoxide dismutase (units/mg protein) in different groups of rats treated with 1 α , 25(OH)₂D₃ during DEN induced rat hepatocarcinogenesis promoted by phenobarbital (mean \pm SE, $n=5$)

Groups	Nodules	Surrounding	Control
A	4.38 \pm 0.56 ^b	5.28 \pm 0.57 ^a	8.64 \pm 0.87
C	6.84 \pm 0.81 ^c	7.60 \pm 0.73	8.84 \pm 0.80
E	4.88 \pm 0.66	5.64 \pm 0.53	8.52 \pm 0.77

Statistical level of significance by using Dunnett's *t*-test. ^b $P<0.01$, ^a $P<0.05$, compared with control. ^c $P<0.05$ compared with group A.

Table 3 Effect of 1 α , 25(OH)₂D₃ on incidence, number and size of hepatocellular lesions during DEN induced rat hepatocarcinogenesis promoted by phenobarbital (mean \pm SE)

Group	No. of rats with nodules	Nodule incidence (%)	Total No. of nodule	Average No. of nodules per nodule bearing liver	Relative size		
					<1mm	>1mm<3mm	>3mm
A	10/10	100	168	16.80 \pm 1.33	94 (55.95)	48 (28.57)	26 (15.47)
C	6/10	60 ^b	22	3.66 \pm 0.88	11 (50.0)	6 (27.27)	05 (22.72)
E	8/10	80 ^d	72	8.00 \pm 1.05	28 (38.88)	24 (33.33)	20 (27.77)

Statistical level of significance by using Dunnett's *t*-test, ^b $P<0.01$, ^d $P<0.01$ compared with group A.

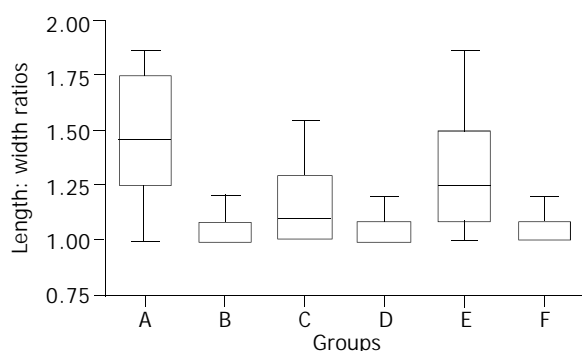


Figure 2 Box-and-whisker plot of the distribution of DNA damage treated with $1\alpha, 25(\text{OH})_2\text{D}_3$ in DEN induced rat hepatocarcinogenesis promoted by phenobarbital.

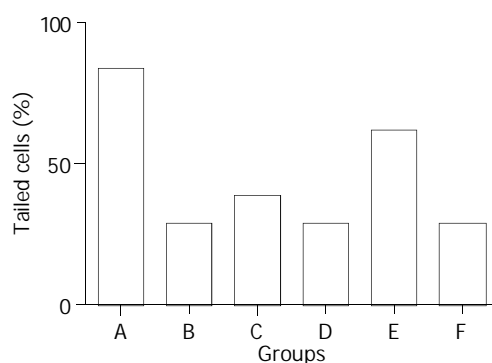


Figure 3 Tailed cells (%) in hepatic cells of rats treated with $1\alpha, 25(\text{OH})_2\text{D}_3$ in DEN induced rat hepatocarcinogenesis promoted by phenobarbital.

Effect of $1\alpha, 25(\text{OH})_2\text{D}_3$ on hepatic cytosolic lipid peroxidation

$1\alpha, 25(\text{OH})_2\text{D}_3$ 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 $1\alpha, 25(\text{OH})_2\text{D}_3$ led to a significant reduction in total MDA production in DEN treated rats. The maximum effect was observed in group C rats whose $1\alpha, 25(\text{OH})_2\text{D}_3$ treatment was started 4 wk before DEN administration and continued till 20 wk, which offered a better protection in group E, in which $1\alpha, 25(\text{OH})_2\text{D}_3$ 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 $1\alpha, 25(\text{OH})_2\text{D}_3$ during DEN induced rat hepatocarcinogenesis promoted by phenobarbital (mean \pm SE, $n=5$)

Groups	Nodules	Surrounding	Control
A	18.72 \pm 1.59 ^b	17.4 \pm 1.34 ^d	2.44 \pm 0.84
C	8.80 \pm 1.02 ^a	7.6 \pm 0.88	3.16 \pm 0.94
E	13.16 \pm 1.23	12.32 \pm 1.03	2.8 \pm 0.79

Statistical level of significance by using Dunnett's t -test. ^b $P<0.01$, ^d $P<0.01$ compared with control; ^a $P<0.05$ compared with group A.

Effect of $1\alpha, 25(\text{OH})_2\text{D}_3$ 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 $1\alpha, 25(\text{OH})_2\text{D}_3$ during DEN induced rat hepatocarcinogenesis promoted by phenobarbital (mean \pm SE, $n=5$)

Groups	Nodules	Surrounding	Control
A	367.6 \pm 25.5 ^b	310.0 \pm 24.9 ^c	226.0 \pm 22.5
C	268.0 \pm 21.5 ^a	254.0 \pm 20.1	248.0 \pm 21.7
E	303.2 \pm 22.5	284.0 \pm 21.6	256.0 \pm 22.5

Statistical level of significance by using Dunnett's t -test. ^b $P<0.01$, ^c $P<0.05$ compared with control. ^a $P<0.05$ compared with group A.

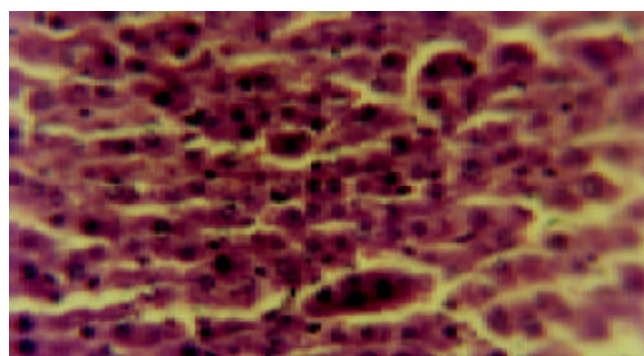


Figure 4 Shows the section of the rat liver (group A) showing abnormal hepatic architecture after a single i.p. Injection of DEN (200 mg/kg b.m.) (HE $\times 325$).

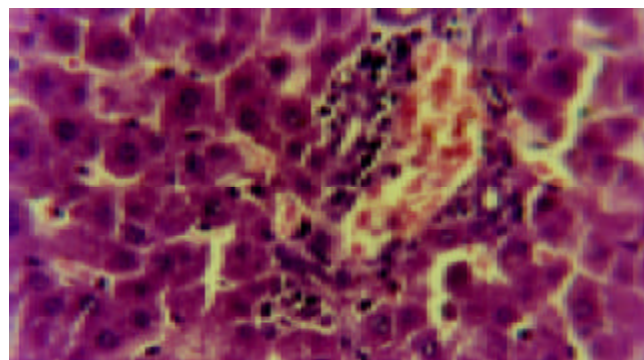


Figure 5 Shows the thin section of normal rat liver untreated (group B) showing hepatocellular architecture (HE $\times 325$).

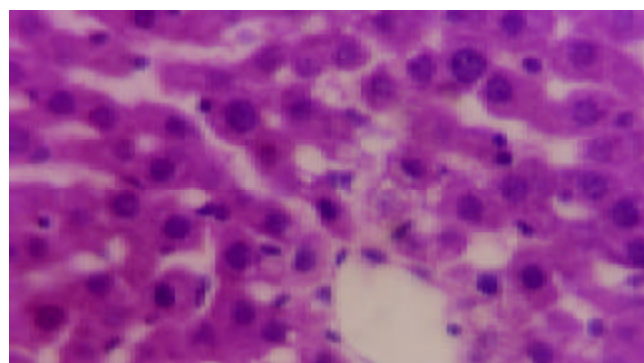


Figure 6 Shows section of rat liver (group C) initiated with DEN and supplemented with $1\alpha, 25(\text{OH})_2\text{D}_3$ [0.3 μg /100 μL propylene glycol per os twice a week for 20 wk] showing almost normal hepatic architecture (HE $\times 325$).

Effect of 1 α , 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 1 α , 25(OH)₂D₃ was found in group B. Groups C and E also showed higher altered activity of 1 α , 25(OH)₂D₃ than the normal control rats. 1 α , 25(OH)₂D₃ was found to be more effective in the inhibition of rat liver carcinogenesis ($P < 0.05$) in abating the GST activity. 1 α , 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 1 α , 25(OH)₂D₃ during DEN induced rat hepatocarcinogenesis promoted by phenobarbital (mean \pm SE, $n=5$)

Groups	Nodules	Surrounding	Control
A	2.52 \pm 0.23 ^b	1.54 \pm 0.23 ^c	0.9 \pm 0.10
C	1.45 \pm 0.27 ^a	1.24 \pm 0.11	1.12 \pm 0.13
E	1.76 \pm 0.29	1.48 \pm 0.19	1.10 \pm 0.11

Statistical level of significance by using Dunnett's t -test. ^b $P < 0.01$, ^c $P < 0.05$ compared with control. ^a $P < 0.05$ compared with group A.

DISCUSSION

The results of our present investigation clearly demonstrated that long term supplementation of 1 α , 25(OH)₂D₃ (at a dose of 0.3 $\mu\text{g}/100 \mu\text{L}$ 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 1 α , 25(OH)₂D₃ in combination with vanadium could effectively inhibit DEN-induced rat liver carcinogenesis^[34-36].

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^[35,38], 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 1 α , 25(OH)₂D₃ could reduce the incidence, multiplicity and size of visible HNs more than 3 mm in size. Preneoplasia (γ -glutamyl transpeptidase -positive and glucose 6-phosphate negative) appeared in 1 α , 25(OH)₂D₃ treated animals 1 wk after carcinogen withdrawal, but livers from 1 α , 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 1 α , 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. 1 α , 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 nucleosome. 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, 1 α , 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 convert 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 1 α , 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 1 α , 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^[36,50-52]. 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]. 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^[50,54]. 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 1 α , 25(OH)₂D₃ abated the production of MDA in different DEN-PB treated rats (groups C and E). The ability of 1 α , 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]. $1\alpha, 25(\text{OH})_2\text{D}_3$ 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- π 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. But the significant decrease ($P < 0.05$) in GST activity was found in group C supplemented with $1\alpha, 25(\text{OH})_2\text{D}_3$. The increased anti-cancer drug resistance of one or more of the GST isoenzymes in resistant cells was compared with normal cells^[64].

$1\alpha, 25(\text{OH})_2\text{D}_3$ 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 $1\alpha, 25(\text{OH})_2\text{D}_3$ may be important for cancer prevention.

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