CHAPTER – II

CHEMOPREVENTIVE ROLE OF 1α,25-DIHYDROXYVITAMIN D₃ ON 2-ACETYLMAMINOFLUORENE (2-AAF) INDUCED RAT LIVER PRENEOPLSIA: STUDIES ON ANTIOXIDANT DEFENSE SYSTEM
Introduction

Induction of the hepatocarcinogenesis by administration of chemicals provides a system for characterizing alterations in the liver at early stages (precursor of malignant tumour formation) process (Farber, 1984). Human HCC and both spontaneous and chemically induced HCC in rodents exhibit considerable similarities with regards to morphology, genomic alterations and gene expression (Feo, et al., 2000) despite their different etiologies. Therefore, investigation of the development of liver cancer in rats and mice might provide valuable insight into the human condition (Thorgersson and Grisham, 2002; Baunasch, et al., 2001). From a toxicological point of view, the liver is of particular interest, since in connection with lifetime bioassays of putative carcinogenesis in rodents, the liver is one of the organs most often affected (Maronpot, et al., 1987). The rat liver is one of the most extensively explored model of carcinogenesis (Saeter and Saglen, 1990; Farber and Sharma, 1987; Goldsworthy et al., 1986). Hepatocytes exhibiting altered morphological and biochemical properties (referred to here as initiated hepatocytes) arise in response to exposure to hepatocarcinogens and the frequency of such initiated hepatocytes can be estimated employing simple immunohistological techniques. Using the methods of quantitative morphometry, the preneoplastic lesions can easily be identified, counted and their number and size can be determined so that the use of suitable chemopreventive agent to alter and / or arrest the multistep hepatocarcinogenesis can be adopted (Moreno et al., 1991; Hida et al., 1994; Rao and Fernandes, 1996). There is substantial evidence indicating that initiated hepatocytes are the precursors of HCC (Bralet, et al., 2002; Sell, 2002; Farber, 1991).

The metabolism of drug has broadly classified into two types: Phase-I (functionalization) and Phase-II (conjugation) reaction. The enzymes for Phase-I reaction introduce a functional group, such as hydroxyl group into the substrate, whereas, the Phase-II enzymes use this functional group as a ‘handle’ for conjugation with such moieties as glucuronic acid, sulphate, GSH, glucose and cysteine yielding a hydrophilic product. These drug-metabolizing enzymes seem to be acting as important biomarkers in characterizing the metabolic patterns of cancer. Currently, attention has
been concentrated on the altered phenotypes during the early phases of cancer development and new markers especially enzymes or isozymes have proved to be useful for the detection of preneoplastic lesions in various organs including liver (Sato, 1989).

The tumour marker enzymes such as glutathione (GSH), and glutathione-S-transferase (GST), have been recognized as important markers in characterizing the metabolic patterns of preneoplastic cells and in the development of strategies for prevention of carcinogenesis and early treatment of cancer (Pitot, 1990). Reduced glutathione a cystein-containing tripeptide, is involved in intracellular protection from free radical damage and has a definite role in detoxifying different xenobiotics, including chemical carcinogens (Chesseaud, 1979; Wattenberg, 1992). GST with the help of GSH, plays an important role in the detoxification of xenobiotic compounds (Nijhoff et al., 1993). The assessment of the toxic response is mainly based on various detoxification pathways, and of these GSH, and GST is the most important molecule (Bhattacharya, 1996)

Antioxidants act either by scavenging on the reactive carcinogenic species or by altering the activity of microsomal mixed function oxidase system. Alteration of this enzyme system of microsomal metabolism by antioxidants could reduce or prevent neoplastic transformation by inhibiting the formation of highly reactive carcinogenic derivatives that react with the cellular macromolecules in many different cell organelles. The induction of alternate metabolic pathway leading to the formation of non-carcinogenic metabolites or the trapping of reactive species by molecule acting as scavengers may also be involved in the anticarcinogenic action of these agents (Prochaska et al., 1985).

In mammalian cells, the intracellular redox balance is maintained by the homeostatic mechanism that links small pools of coenzymes and cofactors to a large redox buffer i.e., thiol system with common chemical properties. Most of the extra and intracellular thiol is represented by the tripeptide glutathione (GSH) [γ-Glu-Cys-Gly] which is principally involved in the protective mechanisms against the deleterious actions of drugs and / or their metabolites (Meister, 1988), acting as a reducing agent and as an
antioxidant, thus combating effectively the pathological conditions that are associated with the formation of free radicals and reactive oxygen compounds (Puri and Meister, 1983).

Glutathione is found to be present in almost all tissues of the body but with a higher concentration in the liver. Recent quantitative cytochemical investigations have proved that hepatocytes within 100µm of the central vein contain much less glutathione than other regions of the lobule (Smith et al., 1979). Three amino acids are mainly required for the biosynthesis of GSH; they are cysteine, glutamic acid and glycine. Studies on rats have shown that (³⁵S) cysteine is rapidly incorporated into liver glutathione and its rate of decay have proved that there are two pools of glutathione – a relatively stable pool containing 3 µmol / g liver with a half-life of 28.5 h and a labile pool containing 4-5 µmol / g with a half-life of 1.7 h (Tateishi and Higashi, 1978). This peptide glutathione plays an important role in protection against tissue damage resulting from exposure to oxidizing environments, such as hyperoxia, hyperbaric oxygen or ozone (Hagen et al., 1986, 1988; Jenkinson, 1988).

Another widely distributed family of enzymes is Glutathione-S-transferase that catalyzes the nucleophilic addition of thiol of the reduced glutathione to a variety of electrophiles (Mannervik, 1985; Armstrong, 1991; Tsuchida and Sato, 1992). This event is thought to aid in preventing the tumourigenic process by eliminating the electrophilic endogenous and exogenous compounds from the body (Habig et al., 1974). Several investigations report’s that many carcinogens and their metabolites have been detoxified by this type of enzymes (Chesseaud, 1979; Morrow and Cowan, 1990). The active sites of GSTs have been shown to contain two binding site for glutathione, G-site and a second site for substrate binding, H-site. The active site might contain either histidine, cysteine, tryptophan, arginine or aspartic acid (Van Ommen et al., 1989, 1991; Tamai et al., 1990; Chang et al., 1991; Ricci et al., 1991). GSTs have been reported to be the reliable markers for preneoplastic lesions and neoplastic tissues in liver, as well as in other organs of the rat placental form (GT-P or GT7-7) and the human placental form (GT-π), the two forms are very similar in physicochemical, enzymatic and immunological properties and are therefore grouped together in the class
Pi under the species-independent classification of GST proposed by Mannervik et al., (1985). A number of investigations suggested the changes in GST isozymes in chemically induced hepatocarcinogenesis of rats showing elevating trends (Kitahara et al., 1983; Sato, 1990) and subsequently GT-p was identified as a good marker for rat hepatic preneoplastic foci (Sato et al., 1984a).

Since cells continuously produce free radicals, their oxidative stress homeostasis can only be maintained if endogenous cellular antioxidants are present. Superoxide dismutase (SOD) eliminates superoxide radical converting it to oxygen and H₂O₂. There are different forms of this enzyme: a Cu, Zn form in the cytosol and the intermembrane mitochondrial compartment (Okado and Fridovich, 2001), a Mn form in the mitochondrial matrix, and another form in the extracellular compartment. The mitochondrial enzyme is essential for life, its decline leading to mitochondrial dysfunction, pathology, or neonatal lethality depending on the level of depletion; MnSOD overexpression can protect against pro-apoptotic or pro-ischaemic insults (Crow and Cruthirds, 2001). SOD alone, however, is not in itself an antioxidant because although it eliminates superoxide radical, it produces another ROS, H₂O₂. Thus, other enzymes must eliminate the hydrogen peroxide produced by SOD and other sources.

Materials and Methods

Animals and Diet

Male Sprague-Dawley rats 80-100g body weight, obtained from Indian Institute of Chemical Biology (CSIR), Kolkata. The animals were acclimatized to standard laboratory conditions (Temperature 24±10°C, RH 55±5% with the altering 12:12 h light-dark cycle) in Tarson cages (4 to 6 rats per cage) for one week before the commencement of the experiment. During the entire period of study, the rats were fed with a semi-purified basal diet (Lipton India Ltd., Mumbai, India) and water ad libitum. All animals shall, strictly be maintained according to the NIH guidelines of care and use of Laboratory animals (CPCSEA Regn. No.: 367/01/C/CPCSEA).
Experimental Regimen

To deduce the cancer chemopreventive potential of vitamin D₃ (VD₃) against 2-acetylaminofluorene (2-AAF) induced rat hepatocarcinogenesis, rats were randomly divided into eight experimental groups (A – D & a – d) as illustrated in fig.2.1, consisting of 10-12 animals in each group. Groups- A, B, C, and D rats were the 2-AAF-treated groups that stared receiving 2-AAF at 9 weeks of age, i.e., from week 4 of experimentation, at a dose of 0.05% in basal diet, once daily for 5 days a week for 16 consecutive weeks, i.e., till week 20. Group-A, the carcinogen control, whereas group-a rats were the untreated normal vehicle control for 2-AAF and VD₃. Group-B rats received VD₃ at a dose of 0.03µg/100µl propylene glycol, per os, twice a week for entire lengths of the study, i.e., for 20 consecutive weeks, starting the treatment 4 weeks before initiation with 2-AAF (long-term continuous study). Treatment of VD₃ in group-C rats was started 4 weeks prior to 2-AAF challenge (at 0 week) and stopped at week 4 from the day of commencement of 2-AAF administration (initiation study). In group-D, VD₃ supplementation at the same dose mentioned above was started 4 weeks after the starting of 2-AAF administration (initiation), i.e., after week 8 and was continued thereafter till the completion of the experiment, i.e., a total of 12 (promotion study). The rats from groups-b, c, and d served as VD₃ controls for groups-B, C, and D respectively, and 12 were received supplementary VD₃ for 20, 4, and 12 consecutive weeks, respectively. Daily food and water intakes were noted and the body weights of the animals from each group were recorded every second day. All the treatments were withdrawn after week 20 and the rats were killed by decapitation between 09:00 and 11:00 h under proper light ether anesthesia after week 21 to carry out experiments. All the animals were fasted over night before being killed.
Fig. 2.1. Basic experimental protocol for morphometry and enzyme assays.

- , Basal diet and drinking water *ad libitum*;
- , Basal diet with 0.05% 2AAF, 5 days a week;
- , Basal diet with 0.03μg VD₃ / 100μl propylene glycol per OS twice a week
- , Basal diet with 0.05% 2AAF and 0.03μg VD₃ / 100μl propylene glycol per OS twice a week
- , Starting treatment
- , Termination of treatment
- , Animals were killed.
Morphology and Morphometry of Liver Tissue

After the rats were sacrificed, their livers were promptly excised, blotted to dry, quickly weighed and examined macroscopically on the surface as well as in 3mm cross sections for gross visible persistent nodules (PNs), which represented focal proliferating, hepatic lesions with a low tendency to spontaneous regression (Farber, 1984). The PNs are easily identified and differentiated from the reddish-brown non-nodular surrounding parenchyma (NNSP) by their grayish-white colour and short demarcation, and the plates of nodules are discontinuous with those of the adjacent liver tissue. The PNs that approximated spheres were measured in two perpendicular directions to the nearest millimeter to obtain an average diameter of each nodule. The PNs were divided into three groups according to their respective diameter and total area of liver parenchyma occupied, namely ≥3, <3->1, and ≤1mm (Moreno et.al., 1991;Bishyae et.al., 1995). The percentage of nodule incidence, total number of nodules and nodule multiplicity were also calculated.

Histopathology of Liver Tissue

After draining the blood, the representative longitudinally excise slices were taken from the left, right-median and right-anterior lobes of the liver. The liver tissue samples were fixed immediately in 10% buffered formalin (to prevent decomposition) solution for fixation, dehydrated with graded ethanol from 50-100%. At least three slices per liver were embedded in low melting point paraffin. Sections of 5 µm in thickness were cut, deparaffinized in xylene and dehydrated with 95, 90, and 70% v/v alcohol. For routine histopathological examination, five contiguous sections were made from each liver slice and were stained by hematoxylin and eosin (H&E) staining (Stewart et.al., 1980). The slices were scanned in different places under a photomicroscope (Adcon,Model No. 5591) for grading the specific hepatocellular lesions according to the published criteria (Bannasch,1976; Stewart et.al., 1980; Moreno et.al., 1991).
Enzyme assays

Preparation of hepatic cytosolic fraction: For biochemical estimation, the animals were fasted overnight and killed under light ether anesthesia. Livers from different group of rats were quickly excised, washed with chilled 0.9% NaCl, blotted, and weighed dry. All subsequent operations were carried out at 0-4°C. The livers were separately homogenized with ice-cold 0.154 M KCl (pH 7.4) by using a precooled Potter–Elvehjem Teflon coated glass homogenizer for 1 min., to make a 10% w/v tissue homogenate. At first the homogenate was centrifuged at 9000-x g for 20 min., and the resultant supernatant fraction was again centrifuged at 105,000-x g (33,000 rpm) for 1 h in a sorvall-OTD-50B ultracentrifuge at 4°C. The supernatant obtained in the second centrifugation from the cytosolic fraction was kept frozen at -20°C until assayed for superoxide dismutase (SOD), glutathione (GSH), and glutathione-S-transferase (GST).

Estimation of GSH: The hepatocytosolic glutathione (GSH) level was estimated by the method of Ellman (1959). In short, 1 ml of cytosol was mixed with 1 ml of 4% 5-sulfosalicylic acid and the mixture was centrifuged at 15,000 x g for 15 min. The supernatant (1 ml) was allowed to react with 9 ml of 0.1 M 5,5’-dithio-bis-(2-nitrobenzoic acid) (Sigma chemical Co., St.Louis, MO, USA) in 0.1 M phosphate buffer (pH 8.0). The reaction mixture was kept at room temperature for 10 min., and read the absorbance at 412 nm by a Hitachi-2000 Spectrophotometer.

Assay of GST activity: The activity of glutathione-S-transferase (GST) in hepatic cytosol was quantified by method of Habig et al., (1974), using 1-chloro-2, 4-dinitro-benzene (CDNB) and GSH (Sigma Chemical Co) as substrates. The reaction mixture (1 ml) consisted of 0.1 M potassium phosphate buffer (pH 6.5), 1 mM CDNB, and 1 mM GSH and a suitable amount of cytosol (1.2 to 1.6 mg protein/ml). The reaction mixture was incubated at 37°C for 5 min., and the reaction was initiated by the addition of CDNB. The increase in optical density was measured spectrophotometrically at 340 nm using a temperature control cell compartment.

Estimation of Manganese-dependent SOD activity: The activity of manganese-dependent SOD (Mn-SOD) was determined as per the method described by Beyer and
Fridovich (1987). The reaction mixture consisted of 50mM potassium phosphate, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 50µM xanthine, 10µM ferricytochrome c, 6nM xanthine oxidase and cytosolic fraction in a total volume of 3.0ml at pH 7.8 and 25°C, the absorbance measured at 550nm. One unit of SOD activity is defined as the amount that causes 50% inhibition of the initial rate of reduction of the cytochrome under the conditions specified. The amount of cytosolic protein was determined by a standard biochemical technique (Lowry et al., 1951).

Statistical Analysis

Data were analyze statistically for differences between the means using Student’s t-test and values of P< 0.05 were taken to imply statistical significance.

Results

During the entire period of study, no differences in food and water consumption were observed among the various groups of animals. Food and water intakes were 10.7-12.8g/100g/day and 8-10ml/day/rat, respectively for all rat groups. Four rats from different experimental groups died before the end of the study (i.e., 20 weeks): two from group-A (16.66%) and one each from groups-C and D (8.33%).

Body and Liver Weight

Table 2.1 shows the final body weight, liver weight and relative liver weight (RLW) of different groups of rats that were killed after 20 weeks of the study. The final body weight of the 2-AAF control (rats group-A) was significantly less (P<0.01) than that of the normal vehicle control (group-a). Supplementation of VD$_3$ (0.03µg/100µl propylene glycol, per os, twice a week) for 20, 4, and 12 consecutive weeks maintained the body weight at the normal level and there was no significant differences between group-a (normal vehicle control) and group-b, c and d (VD$_3$ control), suggesting that VD$_3$ supplementation in this study had practically no adverse effect on the growth
responses of the rats. Treatment with VD$_3$ increased the final body weights of groups-D (promotion group), C (initiation group) and B (long-term continuous group) rats compared to the carcinogen control (group-A), but it showed statistical signification in groups-B (P<0.01) and C (P< 0.05). There was no significant difference among the groups in their liver weights. On the other hand, the RLW of group-A rats was found to be increased significantly (P< 0.02) compared to that of group-a. Although VD$_3$ treatment reduced the RLWs in group-B, C and D as compared with group-A. The result was statistically significant in group-B (P<0.01) and C (P< 0.05). There was no difference in RLWs between normal vehicle control (group-a) and VD$_3$ controls (groups-b, c, and d).

**Effect of VD$_3$ on the number and size distribution of visible hyperplastic nodules in rat liver treated with 2-AAF**

Table 2.2 summarizes the nodular incidence, total number of nodules, and average number per nodule-bearing liver of 2-AAF-treated groups in the presence and absence of VD$_3$. Phenotypically altered hepatocyte populations including persistant nodules (PNs) were found in the livers of 2-AAF-treated groups (i.e., group-A, B, C, and D). There was no visible hepatocyte nodules in the livers of normal control (group-a) as well as VD$_3$ control (groups-b, c, and d) groups. But there was 100% nodular incidence in 2-AAF control rats. Supplementation of VD$_3$ decreased the nodular incidence in 2-AAF-treated groups but the inhibition was maximum (58.34%) in group-B, which received VD$_3$ for 20 successive weeks along with 2-AAF. There was also a decrement in the total number of nodules in all the 2-AAF-treated rats but the reduction was most pronounced in group-B and C. In the VD$_3$-treated rats, the percentage of nodules (≤1mm) was increased compared to the 2-AAF control (18.92%). Also, the nodule multiplicity was decreased significantly in all the 2-AAF-treated groups, e.g., in group-C (P< 0.02) and B (P< 0.01) when compared to the 2-AAF control. The percentage inhibition of nodule multiplicity was also maximum (62.16%) in group-B than in group-C and D when compared to group-A.
**Effect of VD$_3$ on Hepatic Architecture**

Phenotypically altered hepatocyte populations including (PNs) were found scattered in the livers of all 2-AAF-treated groups (i.e., group-A, B, C, and D), but no such alterations were noticeable in untreated normal control (group-a; fig.2.A) or in the VD$_3$ controls (groups-b, c, and d). The H&E-Stained sections of liver slices revealed hepatocellular focal lesions that were clearly distinguishable from the surrounding non-nodular normal parenchyma.

In group-A (fig.2.B,C) a gross alteration in hepatocellular architecture was found and hepatocytes appeared oval or irregular in shape. The altered hepatocyte of foci and nodules were found to be consistently enlarged with more than one nucleus, which were moreover largely vesiculated. Some nuclei in the cells were large and hyperchromatic (Basophilic), indicating prominent centrally located nucleoli. Extensive vacuolation was observed in the cytoplasm around the nucleus with masses of acidophilic (eosinophilic) material and a number of prominent clear cell foci.

In group-C (fig.2.E), i.e., the group received VD$_3$ only for 4 weeks before ‘initiation’ with 2-AAF, a predominance of clear rather than acidophilic cell foci was seen. Cells with two nuclei appeared almost similar to that of normal cells. However, a moderate improvement in vacuolation and compactness of hepatocytes in group-C was evident as compared with group-A, but these improvements over group-A were of lesser extent when compared with the long-term group (group-B). Treatment of rats with VD$_3$ during the ‘promotion phase’ (group-D; Fig.2.F) only marginally improved the hepatocellular phenotype from group-A, though some heterogeneity was still noticeable, and moreover, there was the presence of basophilic cells, scattered in the cytoplasm without being aggregated around the portal vein as observed in group-A. In contrast, the cellular architecture of hepatic lobules seemed to be almost like that of normal liver in group-B (fig.2.D) that received VD$_3$ supplementation during the entire period of study. Liver sections from this group presented only a few clear cell foci. The cells were generally filled with cytoplasmic material and were less vacuolated. The size of the
nuclei was essentially the same as that of normal cells with two nuclei was considerably fewer than in group-A.

**Effect of VD₃ on Hepatocytosolic GSH, GST, and SOD Activities upon 2-AAF challenge**

Table 2.3 shows the levels of GSH, GST, and SOD in different groups of rat livers. From the table, it is evident that, 2-AAF challenge to group-A rats significantly suppressed the level of GSH (P< 0.01) as well as GST (P< 0.001) when compared to the normal vehicle control (group-a). On the contrary VD₃ supplementation to all the 2-AAF-challenged rats (groups-B, C, and D) increased the levels of both GSH, and GST, although the results were significant in groups-B (P< 0.02 and P< 0.001, respectively) and C (P< 0.05 and P< 0.02, respectively) as compared to the carcinogen control. A difference (P< 0.01) between ‘b’ and ‘a’ was also noticed, suggesting that VD₃ supplementation has the capability of GST induction particularly in the long-term phase of carcinogenesis. On the other hand, there was a significant increase (P< 0.001) in the level of Mn-SOD in 2-AAF control rats (group-A) when compared to the normal counterpart (group-a). However, treatment with VD₃ successively reduced the elevated levels of the enzyme but the results were significant in groups-B (P< 0.001) and C (P< 0.001).
Table 2.1: Final body weight, liver weight and relative liver weight of different groups of rats (killed after week 20)

<table>
<thead>
<tr>
<th>Group Treatment</th>
<th>Effective No. of rats</th>
<th>Body Weight (g)</th>
<th>Liver Weight (g)</th>
<th>Relative Weight liver/100g body weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>A 2-AAF</td>
<td>10</td>
<td>253.50±8.43a</td>
<td>8.20±0.51*</td>
<td>3.24±0.15b</td>
</tr>
<tr>
<td>a Normal</td>
<td>10</td>
<td>282.00±5.11</td>
<td>6.20±0.59</td>
<td>2.19±0.21</td>
</tr>
<tr>
<td>B Long term</td>
<td>12</td>
<td>278.3±1.36c</td>
<td>6.50±0.41</td>
<td>2.33±0.12d</td>
</tr>
<tr>
<td>b Long term</td>
<td>12</td>
<td>285.6±2.96</td>
<td>6.20±0.35</td>
<td>2.17±0.31</td>
</tr>
<tr>
<td>C Initiation</td>
<td>11</td>
<td>269.70±1.21e,f</td>
<td>6.70±0.34</td>
<td>2.37±0.12g</td>
</tr>
<tr>
<td>c Initiation</td>
<td>12</td>
<td>282.50±3.95</td>
<td>6.30±0.56</td>
<td>2.23±0.26</td>
</tr>
<tr>
<td>D Promotion</td>
<td>11</td>
<td>260.40±1.43h</td>
<td>7.00±0.41</td>
<td>2.69±0.11</td>
</tr>
<tr>
<td>d Promotion</td>
<td>12</td>
<td>283.60±2.96</td>
<td>6.20±0.48</td>
<td>2.18±0.25</td>
</tr>
</tbody>
</table>

*Values represent Mean ± S.E.(n=10)

a,b,c,d,e,f,g,h P<0.01 and P<0.02 when compared to Normal control (Group a)

c,d,e,f,g,h P<0.01 when compared to Normal control (Group A)

e,g P<0.05 when compared to Normal control (Group A)

f P<0.01 when compared to Initiation control (Group c)

h P<0.01 when compared to Promotion control (Group c)
Table 2.2: Effect of vitamin D3 on the development of nodular hyperplasia in rat liver treated with 0.05% 2-acetylamino-fluorene (2-AAF)

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of rates</th>
<th>Nodule Incidence (%)</th>
<th>Inhibition (%)</th>
<th>Total No of nodules</th>
<th>Nodules relative to size (%) of total number</th>
<th>Nodule# multiplicity</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>≥3mm</td>
<td>&lt;3-&gt;1mm</td>
<td>≤1mm</td>
</tr>
<tr>
<td>A</td>
<td>2-AAF</td>
<td>10/10</td>
<td>100.00</td>
<td>----</td>
<td>37</td>
<td>04 (10.81)</td>
<td>26 (70.27)</td>
<td>07 (18.92)</td>
</tr>
<tr>
<td>B</td>
<td>Long – Term</td>
<td>5/12</td>
<td>41.66</td>
<td>58.34</td>
<td>07</td>
<td>00 (0.00)</td>
<td>02 (28.57)</td>
<td>05 (71.42)</td>
</tr>
<tr>
<td>C</td>
<td>Initiation</td>
<td>6/11</td>
<td>54.54</td>
<td>45.46</td>
<td>12</td>
<td>00 (0.00)</td>
<td>07 (58.34)</td>
<td>05 (41.66)</td>
</tr>
<tr>
<td>D</td>
<td>Promotion</td>
<td>8/11</td>
<td>72.72</td>
<td>27.28</td>
<td>28</td>
<td>02 (7.14)</td>
<td>18 (64.28)</td>
<td>08 (28.57)</td>
</tr>
</tbody>
</table>

# Average no. of nodules/nodule bearing liver

* Values represent Mean ± S.E. (N=10)

aP<0.1, bP<0.02, cP<0.01 when compared with 2-AAF control (Group A)
Table 2.3: Effect of supplemented VD$_3$ on hepatic glutathione (GSH) content, glutathione-S-transferase (GST) and manganese dependent superoxide dismutase (Mn-SOD) activities with or without 2-acetylaminofluorene (2-AAF) challenge

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment(s)</th>
<th>GSH Content (µmol/mg protein)</th>
<th>GST activity (Unit/mg protein)</th>
<th>Mn-SOD activity (Unit/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2-AAF</td>
<td>2.82±0.25$^a$</td>
<td>0.037±0.0027$^b$</td>
<td>8.87±0.40$^b$</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>3.94±0.19$^*$</td>
<td>0.055±0.0035</td>
<td>5.59±0.37</td>
</tr>
<tr>
<td>B</td>
<td>Long-Term</td>
<td>3.81±0.26$^c$</td>
<td>0.052±0.0025$^{d,e}$</td>
<td>5.74±0.36$^d$</td>
</tr>
<tr>
<td></td>
<td>Long-Term Control</td>
<td>3.95±0.21</td>
<td>0.067±0.0020</td>
<td>5.59±0.37</td>
</tr>
<tr>
<td>C</td>
<td>Initiation</td>
<td>3.54±0.25$^f$</td>
<td>0.045±0.0018$^{g,h}$</td>
<td>6.95±0.33$^{i,j}$</td>
</tr>
<tr>
<td></td>
<td>Initiation Control</td>
<td>3.89±0.21</td>
<td>0.057±0.0013</td>
<td>5.60±0.21</td>
</tr>
<tr>
<td>D</td>
<td>Promotion</td>
<td>3.38±0.17$^k$</td>
<td>0.040±0.0014$^l$</td>
<td>8.13±0.45$^l$</td>
</tr>
<tr>
<td></td>
<td>Promotion Control</td>
<td>3.91±0.21</td>
<td>0.060±0.0022</td>
<td>5.57±0.24</td>
</tr>
</tbody>
</table>

$^*$Values represent mean ± SE (n = 10)
$^a$P<0.01, and $^b$P<0.001 when compared to normal control (group-a)
$^c, g$P<0.02, $^f$P<0.05 and $^i$P<0.001 when compared to 2-AAF control (group-A)
$^e$P<0.001 when compared to Long-term control (group-b)
$^b$P<0.001 and $^j$P<0.01 when compared to initiation control (group-c)
$^k$P<0.05 and $^l$P<0.001 when compared to promotion control (group-d)
Fig. 2. A

Fig. 2.A. Contiguous liver sections of rat showing normal hepatocellular architecture (Group-a, normal control) depicting hepatocytes radiating from the central vein. CV-central vein, S-sinusoid and magnification H&E×450.
Fig. 2.B, C abnormal hepatocellular histology (Group A, 2-AAF control) with prominent hyperbasophilic preneoplastic focal lesions and the presence of eosinophilic and clear cell foci after 16 weeks of chronic 0.05% 2-AAF administration. B-basophilic foci, C- clear cell foci, E-eosinophilic cell, PV- portal vein and magnification H&E×450.
**Fig. 2.D.** Almost normal hepatocellular architecture (Group B, VD₃+2-AAF) after 16 weeks of chronic 0.05% 2-AAF feeding along with supplementation of 0.03μg of VD₃ starting 4 weeks before to 2-AAF challenge and continued thereafter, C- clear cell foci, M-mixed cell, S-sinusoid and magnification H&E×450.
Predominance of clear rather than acidophilic cell foci was seen in ‘initiation’ phase (Fig.2E) and marginally improved the hepatocellular phenotype observed in ‘promotion’ phase (Fig.2F) and magnification H&E×450.
Discussion

In the current evaluation the morphometric, antioxidant role of the metabolic enzymes, the chemopreventive role of VD$_3$ has been demonstrated. Cancer chemoprevention refers to the prevention of cancer by intervention using non-toxic chemicals obtained from either natural or synthetic origin before the development of malignancy (Wattenberg, 1992). The neoplastic nodules, which usually progress into HCCs, are the proliferative hepatocellular focal lesions occurring an area larger than one or several liver lobules that disrupt and compress the surrounding parenchyma (Farber, 1973). In most of the experimental models, the focal areas of altered hepatocytes become grossly visible nodules that precede the appearance of malignant tumour. It is assumed that these preneoplastic lesions are the possible precursors of hepatic cancers in rats and also in human beings (Farber and Cameron, 1980). Numerous histopathological and electron microscopic investigations of hepatocarcinogenesis have shown that most neoplastic nodules do not represent a homogenous but a rather heterogeneous cell population comprised of a mixture of different types of altered hepatocytes arranged in an irregular pattern (Bannasch et al., 1978).

Interestingly, the observations obtained from the current evaluation demonstrates the VD$_3$ exhibited pronounced inhibitory effect in the development of visible PNs and smaller number of nodules per nodule-bearing rat liver when compared to 2-AAF control rats Table 2.2. Further, it also reduced the size of PNs by more than 3mm in diameter. Although there are evidence that not all the hepatocyte nodules become malignant during the life span of the animals, numerous observations support the concept that the hyperplastic / neoplastic nodules are the precursors of HCC (Farber and Cameron, 1980; Farber, 1990). In view of this inhibition of nodule incidence and enhancement of their regression by supplementary VD$_3$, shows its important role in cancer chemoprevention. This could be explained in the light of the fact that although the precursor lesions were still present in the livers of VD$_3$ treated rats, their growth rates slowed to such an extent that appearance of PNs was delayed beyond the experimental end point owing to an increased latency period (Farber, 1990).
The results of the present study clearly indicate the hyperplastic nodular hepatocytes formed solid aggregates of one or more cells thick, the prominent ‘hyperbasophilic focal lesions’ mainly around the portal vein in 2-AAF control rats. At least four different types of altered hepatocytes can be identified in the neoplastic nodules of 2-AAF-challenged rat liver (Bannasch, 1976):

a) Clear glycogen storage cells with a disorganization and relative reduction of the granular endoplasmic reticulum.

b) ‘Aidophilic’ glycogen storage cells with a hypertrophy of the agranular endoplasmic reticulum.

c) ‘Vacuolated’ fat storing cells, and

d) ‘Basophilic’ cells, poor in glycogen and rich in ribosomes.

The clear and acidophilic cells primarily developed into “altered hepatocyte foci” (AHF) which are considered to be the small “preneoplastic focal lesions” that leads to malignant transformation in later stages of carcinogenesis with the formation of neoplastic nodules and ultimately HCCs (Peraino et al., 1984). During, the development of neoplastic nodules, the glycogen content of the clear and acidophilic cells is progressively reduced whereas, the number of ribosome increases depicting a distinct hyper basophilic histology, ‘the carcinoma’ of 2-AAF-challenged hepatic tissues (Daoust and Calamai, 1971). Thus, the majority of the neoplastic nodules consist of a mixture of preneoplastic, truly neoplastic and diverse intermediate cells. So it is evident from these observations long-term treatment in reduced hepatocyte aggregation and basophilicity with a reversal of heterogeneity towards normal cytology.

Free radicals generated from intermediates of metabolism are highly reactive, since they contain non-paired electrons. These reactive molecular species are capable of initiating membrane lipid peroxidation, inactivating proteins and enzymes and damaging DNA and RNA, if the cell is insufficiently protected by enzymatic and non-enzymatic antioxidant endogenous non-protein thiol present in animals, which plays an important role in the detoxification of organic xenobiotics, including several chemical carcinogens (Chasseaud, 1979; Uhlling and Wendel, 1992). Because of high nucleophilicity of GSH cys-thiol-sulfur atom, it seems reasonable to postulate that this tripeptide may act as a scavenger of electrophilic species generated by the metabolic
activation of a number of carcinogens, thus eliminating and reducing the concentration of these species available for reaction with cellular macromolecules. Chronic 2-AAF-mediated depletion of GSH might be related to the failure of the system to cope against oxidative stress generated by the accumulation of high amount of toxic metabolites and free radicals in the host that ultimately to cellular DNA damage in vivo. Moreover, this enhanced oxygen tension acted as a signal for MnSOD induction (Skrzydlewska et al., 2001). MnSOD is the first line of defense of the cellular antioxidant system against the oxidative damage initiated 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 (Fridovich, 1983; 1986). VD$_3$ is very much effective in preventing 2-AAF induced changes in hepatocytes possibly through the inhibition of nodular growth, and normalization of 2-AAF induced changes in enzyme activities in rat hepatocarcinogenesis. VD$_3$ may thus be considered as a potential cancer chemopreventive agent for the future, which warrants a detailed molecular mechanistic study.