Carcinogen-induced early molecular events and its implication in the initiation of chemical hepatocarcinogenesis in rats: Chemopreventive role of vanadium on this process

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Abstract

Carcinogen-induced formation of DNA adducts and other types of DNA lesions are the critical molecular events in the initiation of chemical carcinogenesis and modulation of such events by chemopreventive agents could be an important step in limiting neoplastic transformation in vivo. Vanadium, a dietary micronutrient has been found to be effective in several types of cancers both in vivo and in vitro and also possesses profound anticarcinogenicity against rat models of mammary, colon and hepatocarcinogenesis. Presently, we report the chemopreventive potential of vanadium on diethylnitrosamine (DEN)-induced early DNA damages in rat liver. Hepatocarcinogenesis was induced in male Sprague–Dawley rats with a single, necrogenic, intraperitoneal (i.p.) injection of DEN (200 mg/kg body weight) at week 4. There was a significant induction of tissue-specific ethylguanines, steady elevation of modified DNA bases 8-hydroxy-2′-deoxyguanosines (8-OHdGs) ($P<0.0001$; 89.93%) along with substantial increment of the extent of single-strand breaks (SSBs) ($P<0.0001$) following DEN exposure. Supplementation of 0.5 ppm of vanadium throughout the experiment abated the formations of O^6-ethylguanines and 7-ethylguanines ($P<0.0001$; 48.71% and 67.54% respectively), 8-OHdGs ($P<0.0001$; 81.37%), length:width (L:W) of DNA mass ($P<0.01$; 62.12%) and the mean frequency of tailed DNA ($P<0.001$; 53.58%), and hepatic nodulogenesis in preneoplastic rat liver. The study indicates that 0.5 ppm vanadium is potentially and optimally effective, as derived from dose–response studies, in limiting early molecular events and preneoplastic lesions, thereby modulating the initiation stage of hepatocarcinogenesis. Vanadium is chemopreventive against DEN-induced genotoxicity and resulting hepatocellular transformation in rats.
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1. Introduction

Vanadium, a group VB, first transition series, ultra-trace element (molecular weight 50.942) with various oxidation states ranging from −1 to +5, is an endogenous constituent of plants, animals and most mammalian tissues [1,2]. This dietary micronutrient is believed to have a regulatory role in biological systems and is very probably an essential element, just like other 40 essential micronutrients, requiring small amount for normal cell metabolism as well as for proper growth and development of mammals [1–3]. It influences the behaviour of enzymes, regulates the activities of second messengers, signal transduction cascades and carbohydrate metabolism, mimics insulin and growth factor activities, stimulates protein tyrosine kinase and inhibits phosphotyrosine phosphatases and modulates gene expression [4]. This nutritional element has further been considered as a potential agent owing to its ability to prevent regular wear and tear of the genome and accordingly, it is involved in various DNA maintenance reactions and thereby may prevent genomic instability leading to cancers [2,5]. Vanadium compounds have been found to be potentially effective against murine leukaemia, fluid and solid Ehrlich ascites tumour [6], murine mammary adenocarcinoma and HEp-2 human epidermoid carcinomal cells [7] and human carcinomas of lung, breast, and gastrointestinal tract [Köpf-Maier, 1994] [8]. Furthermore, in vivo and in vitro antitumour activities of different vanadium compounds have been

documented by several workers [9–11]. Sakurai et al. [12] have found strong antitumour chemopreventive activities of vanadyl complexes of 1,10-phenanthroline [VO(Phen)$^{2+}$] and related derivatives against human nasopharyngeal carcinoma and the observed effects were found to be superior than the chemotherapeutic drug, cis-diamminedichloroplatinum. Recently, organometallic vanadocene compounds have been found to be potent anti-proliferative agents disrupting bipolar mitotic spindle formation and inducing cell cycle growth arrest in cancer cell lines [13]. Bis(4,7-dimethyl-1,10-phenanthroline) sulfatooxovanadium(IV) or Metvan is equally the most promising multitargeted antitumour vanadium complex with apoptosis-inducing property against human leukemia cells, multiple myeloma cells and a number of solid tumours derived from cancer patients [14]. Again, bisperoxovanadium (bpV) compounds as irreversible protein tyrosine phosphatase inhibitors with broad-spectrum antineoplastic activities are also under investigation [15].

A series of studies from our laboratory has shown that supplementation of 0.5 ppm vanadium in drinking water was quite effective in suppressing chemical hepatocarcinogenesis in rats without any toxic manifestations. Reports indicate that the anti-tumour effect of 0.5 ppm vanadium may be mediated through selective induction and stabilization of hepatic xenobiotic biotransforming enzymes [16], inhibitions of γ-glutamyl transpeptidase (GGT)- [17] and placental glutathione S-transferase (PGST)-positive foci [18], suppression of 2-AAF-induced DNA–protein crosslinks formation [19] and reduction of proliferating cell nuclear antigen immunoreactivity [20] in preneoplastic rat liver. The 0.5 ppm (4.27 μmol/L) concentration of vanadium was chosen exclusively by dose–response studies made in our laboratory much earlier in untreated normal rats with respect to inductions of hepatic glutathione (GSH) and Glutathione S-transferase (GST) activity [21]. This particular dose of 0.5 ppm has been found to be well tolerated and suitable with adequate growth responsive effect. Vanadium at this concentration was also found to be devoid of toxicity, since no histological abnormalities or histopathological changes were noticed neither in the liver and kidney nor in the stomach of the rats studied [21]. In this paper, we have further detailed the dose–response studies of vanadium, which we have made in recent times with respect to carcinogen-induced DNA damage and development of preneoplastic lesions (nodulogenesis) in Sprague–Dawley rat liver chemical carcinogenesis model.

We preferred rat model over mouse, because: first, many mouse strains seem to yield high rates of spontaneous liver tumours. Spontaneous liver tumours in rats are more rare [22]. High background incidence of tumours in mice liver may also confound the interpretation of dose response in chemical carcinogenicity studies in murine liver [23]; second, rat liver is a frequent target for the development of chemically induced cancer in rodents, and it is the most commonly used experimental model for investigating multistage carcinogenesis in vivo [24]. Furthermore, in the rat liver carcinogenesis models, a variety of enzyme-altered condition has been studied for their relevance to preneoplastic and neoplastic developments. For example, an immunohistochemically demonstrable enzyme

marker, PGST has been utilized for the identification of liver preneoplastic focal lesions. In contrast, no equivalent markers for preneoplastic foci are available for mice [25]. In addition, rats have got several other advantages [26], like (a) the many similarities between the rat and human metabolic pathways; (b) the many similar anatomical and physiological characteristics that allow for comparisons in pharmacokinetics; (c) the short life span, especially for carcinogenesis study, which allows observation of DNA transformation from its initial stage to full-blown malignancy; (d) high levels of natural killer (NK) cell immunity; and (e) the easy availability, ease of breeding, and the existence of a large database to enable comparison of present to reported literature findings.

Presently we extend our study further to have insights into the early molecular events associated with the 'initiation' of carcinogenesis in order to have an understanding of the underlying basis of chemopreventive potential of vanadium in modulating the initiation event and thereby limiting DNA damage in vivo. Experimental hepatocarcinogenesis can be induced by various chemical carcinogens, such as, diethylnitrosamine (DEN), 2-acetylaminofluorene (2-AAF), aflatoxin B1. DEN is a potent hepatocarcinogen in rats influencing the initiation stage of carcinogenesis during a period of enhanced cell proliferation induced by hepatocellular necrosis and forming DNA–carcinogen adducts, inducing DNA-strand breaks and in turn hepatocellular carcinomas (HCCs) without cirrhosis through the development of putative preneoplastic focal lesions [27]. Formation of DNA–carcinogen adduct is therefore a prerequisite for chemical carcinogenesis. Besides, carcinogen-induced alterations of the DNA helix include helical distortion, oxidative base modifications, single-strand and double-strand breaks, DNA–DNA inter-strand as well as DNA–protein crosslinks and chromosomal aberrations, and as such carcinogen-induced DNA damage has been implicated as one of the early steps in chemical carcinogenesis [28]. Among the most abundant and mutagenic oxidative base modifications, 8-hydroxy-2′-deoxyguanosine (8-OHdG), produced by the oxidation of deoxyguanosine is considered as the most sensitive and potential marker of oxidative DNA damage [29]. It has been shown that 8-OHdG is closely associated with certain diseases, including cancer, and is produced in various experimental models of chemical carcinogenesis [30,31]. Thus, studying the pattern of changes in the levels of tissue-specific DEN-induced alkylated DNA adducts as well as 8-OHdGs following carcinogen assaults could be quite relevant in understanding the 'initiation' event of carcinogenesis. ^{32}P-post-labeling and high performance liquid chromatography (HPLC) provide sensitive techniques to quantify respectively ethylated DNA bases and oxidative bases in tissue DNA samples. Besides 8-OHdGs, the magnitude of DNA single-strand breaks (SSBs) is a measure of genotoxicity following carcinogen exposure. The inability of cells to repair such damage adequately is a putative causal event in chemical carcinogenesis [31,32]. Single Cell Gel Electrophoresis (SCGE) or the Comet assay, in particular the alkaline version of the assay, has become a popular method for the sensitive analysis, detection and quantitation of genotoxic DNA damage caused by various chemical and physical agents.

Damage is detected as DNA strand-breaks, alkali-labile damage and excision repair sites in individual interphase cells [33].

The principal aim of the study therefore is to find out the possible mechanism by which vanadium exerts antigenotoxic and thereby anti-hepatocarcinogenic effects in vivo. The study focuses the interaction of the dietary micronutrient vanadium with the critical molecule like DNA and inhibition of early DNA damages by this trace metal in modulating the initiation event of carcinogenesis. Thus, the present study was designed with two basic objectives: first, to assess the roles of DNA adducts, 8-OHdGs and SSBs on the hepatocarcinogenesis initiation; and second, to find out the chemopreventive potential of the optimum and effective dose of vanadium through dose–response studies in limiting the extent of molecular markers and resulting expression of premalignant phenotype in a rodent model of experimental hepatocarcinogenesis.

2. Materials and methods

2.1. Materials and maintenance of animals

All the reagents and biochemicals, unless otherwise mentioned were obtained from Sigma Chemicals Co. (St. Louis, MO) and E. Merck (Franfurter Straβe, Darmstadt, Germany).

Male Sprague–Dawley rats obtained from the Indian Institute of Chemical Biology (CSIR), Kolkata, India weighing 80–100 g at the beginning of the experiments were used throughout the study. The animals were acclimatized to standard laboratory conditions (temperature $24 + 1$ °C, relative humidity $55 + 5\%$ and a 12 h photoperiod) and were housed in Tarson Cages (four to five rats per cage) for 1 week before the commencement of the experiment. During the entire period of study, the rats were supplied with a semi-purified basal diet (Lipton India Ltd., Mumbai, India) and water *ad libitum*. The recommendations of Jadavpur University's "Institutional Animal Ethics Committee" ["Committee for the Purpose of Control and Supervision of Experiment on Animals" (CPCSEA Regn. No. 0367/01/C/CPCSEA) INDIA] for the care and use of laboratory animals were strictly followed throughout the study and the particular project was approved by the Chairman of the Committee.

2.2. Experimental regimen

Different sets of rats were randomly divided into four experimental groups as illustrated in Fig. 1 for carrying out molecular studies (short-term studies). Groups C and D rats were the DEN-treated groups that received a single, necrogenic, intraperitoneal (i.p.) injection of DEN (200 mg/kg body weight in 0.9% saline) at 9 weeks of age i.e. at week 4 of experimentation, whereas groups A and B rats were the normal and vanadium controls respectively. Group C rats were the DEN control; while Group B (vanadium control) and D (vanadium+DEN) rats received 0.5 ppm (4.27 μmol/L) vanadium as ammonium metavanadate (NH_4VO_3,+V oxidation state) in terms of the salt weight in drinking water, *ad libitum* starting 4 weeks prior to DEN initiation and stopped at week 4 prior to DEN initiation. Solutions of vanadium (pH 7.0) were renewed
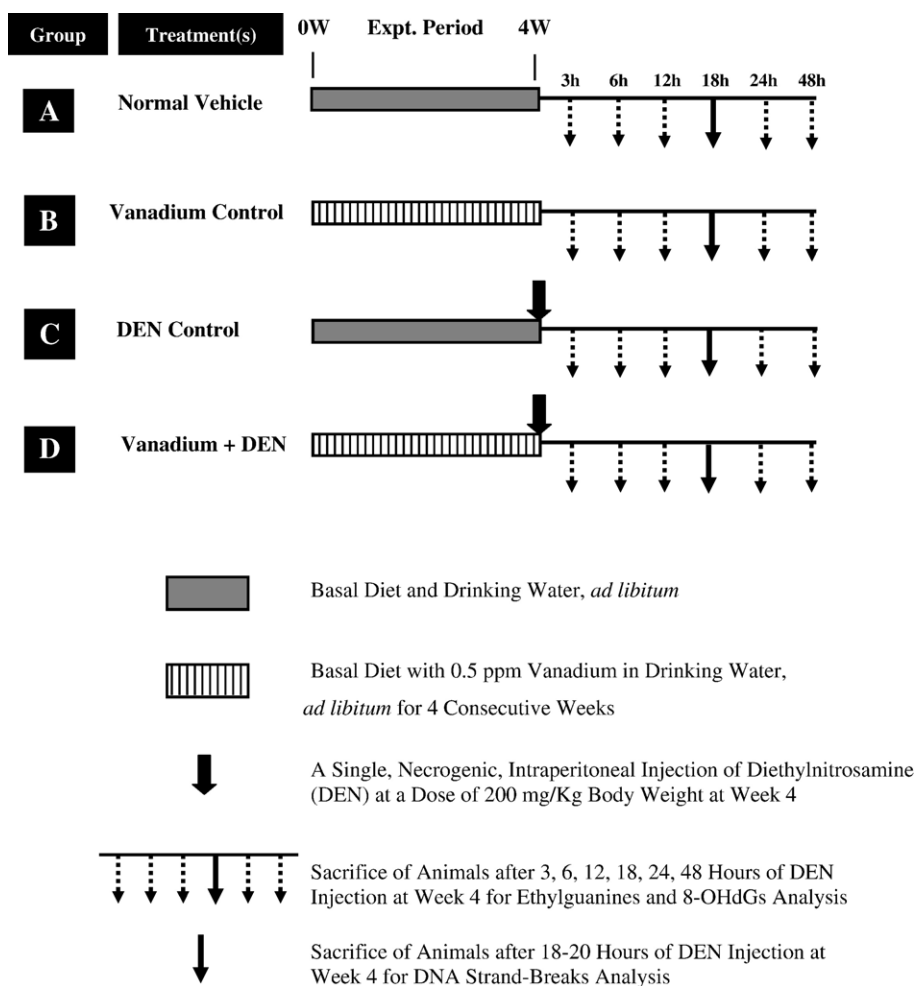

Fig. 1. The basic experimental regimen for molecular studies.

every 2–3 days. Daily food and water intakes were noted and the body weights of the animals from each group were recorded every second day. All the rats were sacrificed by decapitation between 0900 and 1100 h under proper light ether anesthesia after week 4 at various time points to carry out experimentations. All the animals were fasted over night before sacrifice. For the estimation of ethylguanines and 8-OHdGs, rats were sacrificed after 3, 6, 12, 18, 24, and 48 h and for DNA strand-breaks after 18–20 h of DEN injection; livers were promptly excised and hepatic DNA was isolated. For evaluation of preneoplastic lesions, all the four groups of rats were sacrificed after week 21 (long-term study), maintaining groups B and D rats with 0.5 ppm of vanadium for 20 consecutive weeks and DEN administration to groups C and D rats at week 4. Following 3 weeks of recovery, all the DEN-initiated rats were given phenobarbital (PB) (0.05% in basal diet), 5 days a week till week 20.

2.3. Dose–response studies of vanadium

Dose–response studies of vanadium were carried out in the group D rats in two time courses: (a) short-term study using in vivo DNA damage by the alkaline Comet assay and (b) long-term study involving evaluation of preneoplastic lesions. We have used seven different concentrations (μmol/L) (0.5, 1, 2, 4, 6, 8, and 16) of vanadium for both the studies, a and b. The detail of the Comet assay is given in Section 2.7. For the rats sacrificed after 21 weeks, the livers were promptly excised, blotted, and weighed. The livers were then examined macroscopically on the surface as well as in 3-mm cross sections for gross visible persistent nodules (PNs), which represent focal proliferating, hepatic lesions with a low tendency for spontaneous regression [34]. The PNs were easily identified from the reddish-brown non-nodular surrounding parenchyma (NNSP) by their greyish-white color which clearly differentiated them from the adjacent liver tissue.

2.4. Histopathology

After draining the blood, liver slices were taken from each lobe of the liver. The tissue slices were at once immersed in 10% buffered formalin solution for fixation, dehydrated with graded ethanol solutions from 50% to 100%, and then embedded in paraffin. Sections of 5 μm in thickness were cut and stained with haematoxylin and eosin [35]. The histopathological slides were observed under an ADCON 5591 (ADCON, Cleveland, USA) photomicroscope. All the slides were examined without prior knowledge of the treatment given to the animals from which the tissue samples under investigation were taken.

2.5. Analysis of tissue-specific DNA adducts

The tissue DNA samples (5 μg) were enzymatically digested to deoxynucleoside 3′-monophosphates with 0.21 U micrococcal nuclease and 0.174 U spleen phosphodiesterase at 37 °C for about 4.5–5 h [36]. After treatment of DNA samples with 5 μg nuclease P1 for 30 min at 37 °C, the hydrolysate enriched in adducted nucleotides, viz., O^6-Ethylguanine (O^6eGua) and 7-Ethylguanine (e^7Gua) was then labeled by incubation with 24 μCi carrier-free [γ-^{32}P]ATP (3000 Ci/mM) and 10 U T4 polynucleotide kinase at 37 °C for 30 min in 25 μL of bicine buffer mixture [36,37]. Resolution of ^{32}P-labelled digests treated with nuclease P1 was carried out by multidirectional polyethyleneimine-cellulose Thin-Layer Chromatography (TLC) fingerprinting using the contact transfer technique [38]. The solvent systems selected were: 1 M sodium phosphate, pH 6.8; 4 M lithium formate, 7.5 M urea, pH 3.5; 0.65 M LiCl, 0.45 M Tris–HCl, 7.7 M urea, pH 8.0; 1.7 M sodium phosphate, pH 5.0. Analysis of total nucleotides was achieved by direct ^{32}-P labeling of another aliquot of the DNA digest mentioned as above, followed by one-dimensional TLC of the labeled digest. The adduct spots were detected by autoradiography from 72 to 96 h at −80 °C using Kodak XAR-5 films and intensifying screens. DNA adduct concentration was estimated from the measurement of radioactivity in adduct as well as in total nucleotides and the result was expressed as fmol of adducts/μg DNA, considering 1 μg of DNA=0.3×10^7 fmol nucleotides [39].

2.6. Measurement of 8-OHdGs

DNA was extracted from perfused livers of different groups of rats with chloroform only (because phenol exposure would artificially increase

oxidative base concentration in DNA samples) following the protocol of Dahlaus and Appel [40] with minor modifications. Briefly, one volume of nuclear fraction obtained from liver homogenate by centrifugation was mixed with eight volumes of extraction buffer (1 M NaCl, 10 mM Tris–HCl, 1 mM EDTA, 2% SDS, pH 7.4) and one volume of chloroform:isoamyl alcohol (12:1 v/v). After vigorous shaking, the aqueous phase was separated by centrifugation and DNA in TE buffer was incubated with a mixture of RNase T_1 and RNase A. Finally, DNA was extracted again and precipitated with chilled ethanol. DNA concentration was estimated spectrophotometrically using 20 A_{260} U/mg.

200 μg of DNA (4–5 A_{260} U/200 μL) in 40 mM Tris, pH 8.5, containing 10 mM $MgCl_2$ was first denatured by heating at 95 °C for 3 min and then cooled on ice. The extracted DNA was digested into deoxynucleosides by incubation for 2 h at 37 °C with a mixture of DNase I (from bovine pancreas; 200 U/mg DNA), spleen exonuclease (0.01 U/mg), snake venom exonuclease (0.5 U/mg), and E. coli alkaline phosphatase (10 U/mg) [41]. The incubation was terminated with acetone to precipitate proteins and DNA was dissolved in distilled, deionized water.

The content of 8-OHdGs in the digested DNA was measured by an electrochemical detector (ECD) coupled with high performance liquid chromatography (HPLC-ECD system) [42] which consisted of a Waters 600E pump, a Whatman Partisphere-5 C_{18} column and a UV detector (Hewlett-Packard 1050, 254 nm) connected to an ECD (Hewlett-Packard 1049A) in series for monitoring deoxyguanosines (dGs) and 8-OHdGs respectively. The mobile phase consisted of 10 mM $NH_4H_2PO_4$, 10 mM KCl, 1 mM EDTA and 10% aqueous methanol (pH 4.7) and the flow rate was set at 1.0 ml/min. The content of 8-OHdGs in each DNA sample was expressed as the molar ratio of 8-OHdG $\times 10^5$ to total dGs based on the peak height of authentic 8-OHdG with EC detector and the UV absorbance at A_{254} of dGs [42]. On prolonged incubation of the DNA sample with E. coli alkaline phosphatase under the conditions described above, the content of 8-OHdGs increased at the rate of 0.4+0.1 residue/10^5 dG/h. Therefore this blank value for 8-OHdG was subtracted from the observed data. In Fig. 4, background 8-OHdG levels in control DNA (0.8–1.2 residue/10^5 dG) were subtracted.

2.7. Estimation of in vivo DNA strand-breaks by Comet assay

In vivo DNA strand-breaks were measured in liver samples using the alkaline Comet assay, essentially as described by Olive et al. [33]. The tissues were homogenized in phosphate-buffered saline (PBS; pH 8.0) under refrigeration and filtered. Cell viability was determined by the trypan blue method. 4 μL of the homogenized tissue was then transferred to 50 μL of fresh PBS (pH 7.5), washed, suspended in 150 μL of 1% low melting point agarose at 37 °C, and pipetted onto an agarose-precoated glass microscope slide. Slides were prepared in triplicate. The slides were immersed for 60 min in freshly-prepared ice-cold lysis solution (2.5 M NaCl, 0.1 M Na_2EDTA, 10 mM Tris–HCl (pH 10), 10% DMSO and 1% Triton X-100) at 4 °C in the dark, washed, and then subjected to horizontal electrophoresis using freshly made buffer (0.3 M NaOH and 1 mM Na_2EDTA, pH>13). After electrophoresis, the slides were stained with 5 μg/ml ethidium bromide and viewed under a Zeiss fluorescence microscope equipped with a green excitation filter and a 590 nm barrier filter. Routinely 150 cells (50 cells/slide) were screened per liver sample from each animal and then the mean value and standard error of mean (SEM) were calculated from the 15 values obtained from 15 rats under experimentation. Nucleoid DNA extends under electrophoresis to form 'comet tails', and the length of the comets was evaluated for determination of the percentage of tailed DNA. This value is linearly related to the frequency of DNA breaks [33].

2.8. Statistical analysis

The data were analyzed using the GraphPad Prism software package, Version 4.01 (Barcode Softwares, Baltimore, MD). Student's t-test was performed to compare sample means and the results were expressed as Mean+S.E. One-way ANOVA followed by Tukey–Kramer multi-comparison test was also performed to evaluate the changes among different time intervals within a variable using the error calculated from ANOVA. Statistical significance was set at $P<0.05$ for all the values.

3. 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 10.7–12.8 g 100 g^{-1} day^{-1} and 8–10 ml/day/rat respectively for all rat groups.

3.1. Determination of optimum dose of vanadium from dose–response studies

Fig. 2 reveals the dose–response curve of vanadium at 7 specified doses in relation to DEN-induced early DNA damage in the preneoplastic liver of group D rats. The pattern of the curve shows that, there was almost 82% of tailed DNA in the liver of the rats as measured by the in vivo Comet assay following 4 weeks of 0.5 μmol/L of vanadium pre-treatment. Gradual increase in vanadium concentration (up to 4 μmol/L) resulted in an almost linear decrease in the frequency of tailed DNA. At 4.27, 6 and 8 μmol/L of vanadium concentrations, a steady maintenance of the dose–response curve was noticed at these points and no further reduction/protection in DNA damage by supplementary vanadium was observed. This indicates that, vanadium at these concentrations exerts optimum chemoprotective effect in vivo. However, further rise in vanadium concentration beyond 8 μmol/L (e.g. 16 μmol/L) shows (slightly) elevated pattern in the nature of the curve, which suggests that vanadium treatment beyond 8 μmol/L may become cytotoxic.

Again, from Table 1, it is further evident that vanadium treatment for 20 consecutive weeks in a long-term experimental regimen at the concentration of 4.27 μmol/L exerts optimum chemopreventive effect in inhibiting hepatic preneoplastic lesions in terms of nodule incidence and total number. Further, with this particular dose of vanadium, there were no toxicologically significant changes in hematology, clinical chemistry and clinical enzymology, body and relative organ weights etc. Based on these findings, we have used 4.27 μmol/L

Table 1
Dose–response studies of vanadium with respect to nodular hyperplasia in rat liver treated with diethylnitrosamine (200 mg/kg body weight) and phenobarbital

Vanadium concentrations (μmol/L)	No. of rats with nodules	Nodule incidence (%)	Total no. of nodules	Nodule [a] multiplicity
0.5	15	100.00	167	11.13±2.76 [b]
1.0	13	86.66	139	10.70±1.68
2.0	9	60.00	82	9.11±3.64
4.0	4	26.66	17	4.25±1.48
6.0	4	26.66	17	4.25±1.59
8.0	5	33.33	21	4.20±2.82
16.0	9	53.33	76	8.44±2.71

[a] Average number of nodules/nodule bearing liver.
[b] Values represent Mean±S.E. ($n=15$).

or 0.5 ppm as the optimum, well tolerated and pharmacologically effective dose of vanadium in our study.

3.2. Histopathological profile of liver

Phenotypically altered hepatocyte populations including persistent nodules (PNs) were found scattered in the livers of DEN-PB-treated groups (i.e., groups C and D); but no such alterations were noticeable in untreated normal control (group A; Fig. 3A) or in the vanadium control group (group B) (figure not shown). In group C rats (Fig. 3B, C), a gross alteration in hepatocellular architecture was found and hepatocytes appeared oval or irregular in shape. The altered hepatocytes of foci and nodules were found consistently enlarged with more than one nucleus, which were moreover largely vesiculated with centrally located nucleoli. Some nuclei in the cells were large and hyperchromatic (basophilic) (Fig. 3C), indicating prominent hyperbasophilic preneoplastic focal lesions around the portal vein that were clearly distinguishable from the NNSP. 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 contrast, the cellular architecture of hepatic lobules seemed to be almost like that of normal liver in group D (Fig. 3D) that received 0.5 ppm of vanadium supplementation during the entire period of study, i.e. for 20 consecutive weeks. 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 and cells with two nuclei were considerably fewer than in group C rats.

3.3. Effect of vanadium on the levels of formation of ethylated DNA adducts in rats induced by DEN at different time points

Fig. 4 shows the kinetics of formation and persistence of ethylated DNA adducts (O^6eGua and e^7Gua) in rat liver after a single, necrogenic, i.p. injection of DEN at sequential time points. The levels of ethylguanine formation in rat liver DNA have been plotted as a function of time following carcinogen assault. The formation of O^6eGua (Fig. 4A) and
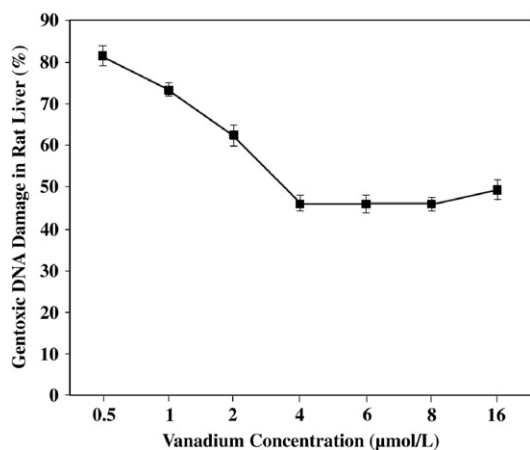
Fig. 2. Dose–response curve defines the relationship between vanadium concentrations (μmol/L) and genotoxic DNA damage (%) following a single, necrogenic dose (200 mg/kg body weight) of diethylnitrosamine (DEN) injection in rat liver. Each bar indicates Mean±S.E. [$n=15$].
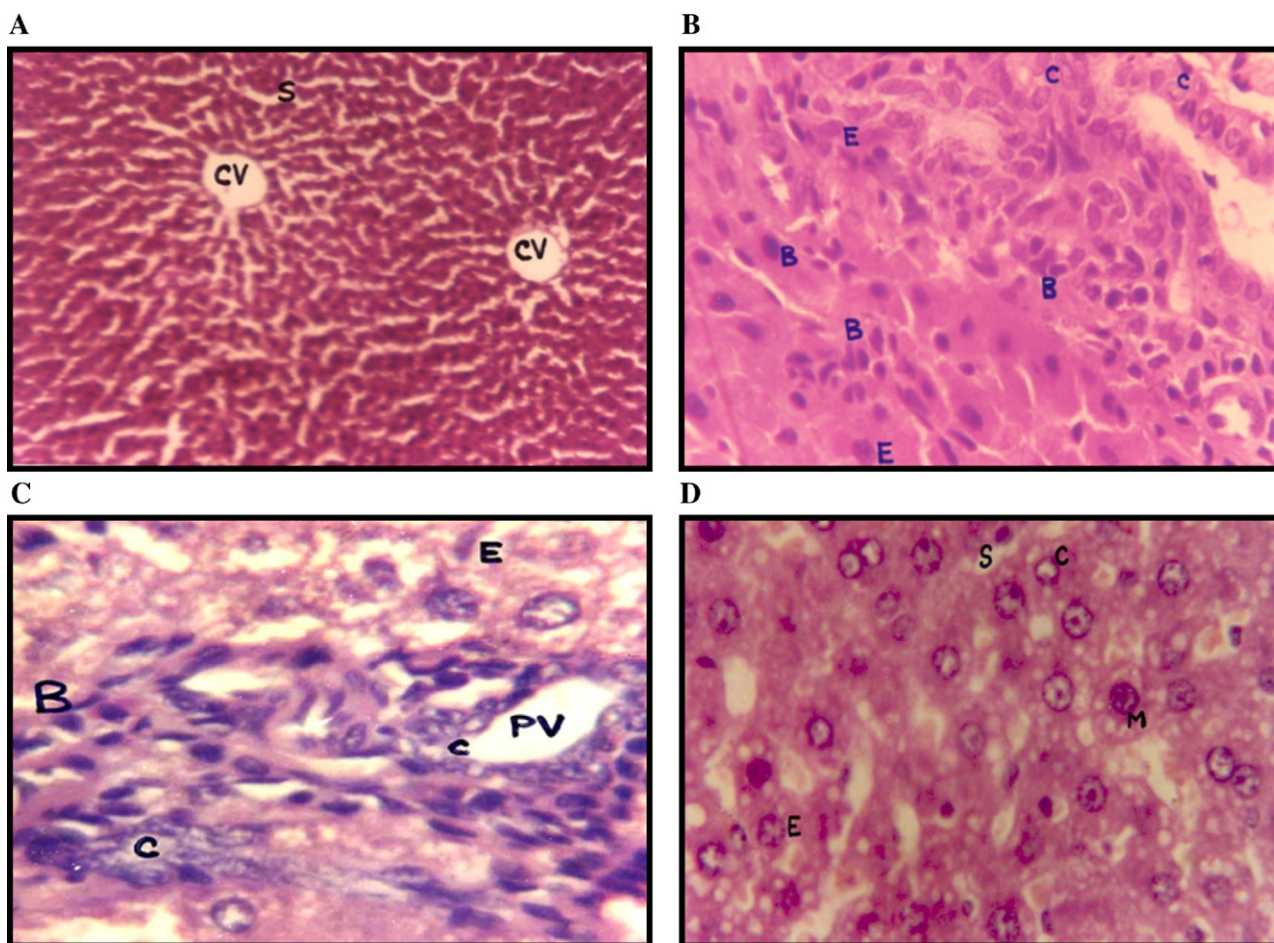
Fig. 3. Contiguous liver sections of rats being sacrificed after 21 weeks, showing (A) normal hepatocellular architecture (Group A, normal control) depicting hepatocytes radiating from the central vein; (B, C) aberrant hepatocellular phenotype (Group C, DEN+PB Control) with prominent hyperbasophilic preneoplastic focal lesions and the presence of eosinophilic and clear cell foci following a single, necrogenic, inraperitoneal injection of diethylnitrosamine (DEN) (200 mg/kg body wt.) at week 4 and application of phenobarbital (PB) (0.05% in basal diet) thereafter on and from week 8; and (D) almost normal hepatocellular architecture (Group D, Vanadium+DEN+PB) following DEN+PB regimen along with continuous supplementation of 0.5 ppm vanadium starting the application 4 weeks before initiation and continued till 20 weeks. B—Basophilic Foci; CV—Central Vein; C—Clear Cell Foci; E—Eosinophilic Cell; M—Mixed Cell; PV—Portal Vein; S—Sinusoid. Mag. (A) H&E ×150; (B–D) H&E ×450.

e^7Gua (Fig. 4B) in DNA was rapid with both ethylguanines apparently formed simultaneously over the given time periods. The time dependency of hepatic DNA alkylation to reach a maximal level ranged from 3 to 18 h following DEN exposure. After 18–20 h, the rate of formation of ethylated adducts started to decline in both the DEN-treated groups (groups C and D), indicating a prominent role of vanadium against adduct formation. There was a significant reduction ($P<0.0001$) in O^6eGua level in vanadium-supplemented rat liver (group D) at 18 h (21.25%), 24 h (26.87%) and 48 h (48.71%) when compared to DEN control. However in case of e^7Gua, vanadium treatment caused a significant reduction ($P<0.0001$) in adduct level at 48 h (67.54%). Ethylguanines were not detected in Groups A (normal control) and B (vanadium control). One way ANOVA followed by Tukey–Kramer multi-comparison test showed significant changes in the pattern of O^6eGua and e^7Gua adduct levels during six sequential time points studied herein (i.e., after 3, 6, 12, 18, 24 and 48 h of DEN injection)

in group C (DEN control) [$P<0.0001$; Computed F is much more greater than the Critical $F_{0.05\ (2.32)}$ which amounts to 289.99 and 279.97 for O^6eGua and e^7Gua respectively; table not shown] as well as in group D (vanadium+DEN) [$P<0.0001$; Computed F is much more greater than the Critical $F_{0.05(2.32)}$ which amounts to 292.30 and 132.66 for O^6eGua and e^7Gua respectively; table not shown].

3.4. Effect of vanadium on the levels of formation of 8-OHdGs in rat liver induced by DEN at different time points

The formation of 8-OHdGs in DNA was significant and rapid and the time dependency of hepatic DNA base lesion to reach a maximal level ranged from 3 to 18 h following DEN exposure ($P<0.0001$; 89.93%) (Fig. 5) when compared to group A. After that, there was a steady maintenance of oxidative adducts in the carcinogen control group (group C). On the other hand, there was a significant reduction in 8-OHdG levels in vanadium supplemented rat liver (group D) at 18 h ($P<0.02$; 28.03%),
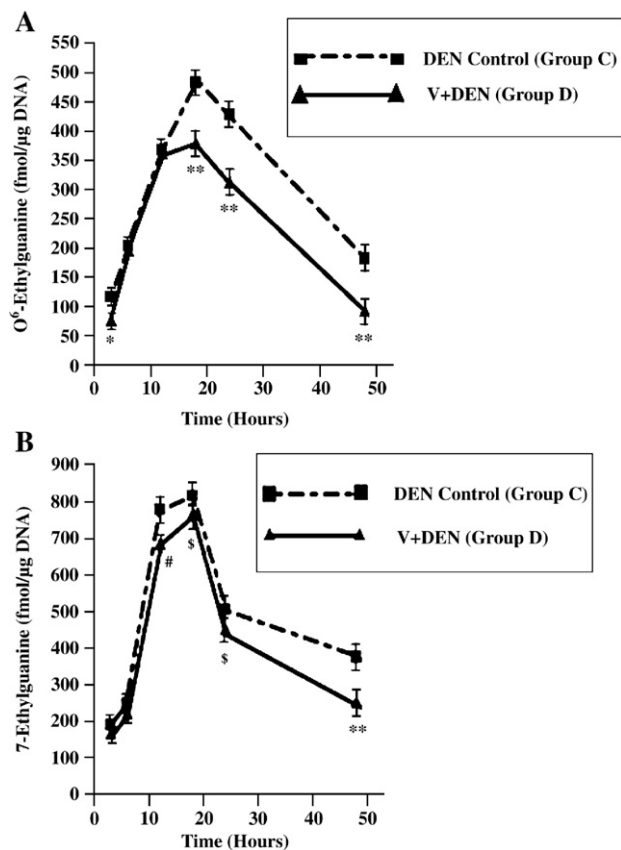
Fig. 4. Levels of formation and persistence of ethylated DNA adducts, namely (A) O^6-ethylguanine (O^6eGua) (B) 7-ethylguanine (e^7Gua) in rat liver at sequential time points in presence (Group D) or absence (Group C) of 0.5 ppm vanadium supplementation following a single, necrogenic dose (200 mg/kg body weight) of diethylnitrosamine (DEN) injection. Each bar indicates Mean ± S.E. [n = 15]. (A) *P < 0.01 at 3 h and **P < 0.0001 at 18, 24 and 48 h; and (B) #P < 0.001 at 12 h, $$P$ < 0.05 at 18 and 24 h and **P < 0.0001 at 48 h after DEN injection when compared to DEN Control (Group C).

24 h (P < 0.001; 48.58%) and 48 h (P < 0.0001; 81.37%) when compared to DEN control. One-way ANOVA showed significant changes in the pattern of 8-OHdG levels [P < 0.0001; Computed F is much greater than the Critical $F_{0.05\ (2.32)}$ which amounts to 55.17 (group C) and 232.29 (group D); table not shown] during six sequential time points, which suggests a prominent role of 8-OHdG in carcinogenesis and its modulation by vanadium.

3.5. Effect of vanadium on hepatic DNA damage

The mean length to width (L:W) ratio of the DNA mass indicating the extent of DNA damage was increased in the carcinogen control group (Group C) in comparison with the normal control (Group A) (P < 0.0001; Fig. 6A). There was also a significant increase in the frequency of tailed DNA in Group C rats compared to Group A rats (P < 0.0001; Fig. 6B). A short-term treatment with vanadium (Group D) reduced the L:W ratio of DNA mass (62.12% reduction; P < 0.01) and the mean frequency of tailed DNA (53.58% reduction; P < 0.001) compared to that of Group C carcinogen control rats.

4. Discussion

We report here the chemopreventive potential of low dose of vanadium in inhibiting DNA adducts and preventing oxidative DNA damages during the early stages of hepatocarcinogenesis in rats. Vanadium supplementation has been found to inhibit the formation of ethylguanines, viz., O^6eGua and e^7Gua, tissue-specific oxidative DNA bases 8-OHdGs, and SSBs in hepatocytes. The results thus indicate that, vanadium at the 0.5 ppm dose, as derived from dose–response studies exerts potential antigenotoxic effect against DEN-induced initiation of hepatocarcinogenesis by inhibiting the formation of DNA adducts and resulting DNA damage in vivo.

0.5 ppm vanadium that we have used is a "low dose" of vanadium. It is mentioned in the literature that at least 5 ppm vanadium in drinking water for entire the life-term is absolutely devoid of any symptoms or signs of toxicity [43] which is 10 times greater than the dose used in our present study. Moreover, vanadium at a dose of 6 mg V/kg diet (120 μmol V/kg) administered to Wistar rats in drinking water did not produce any toxicity [44] and also no adverse symptoms were noted in rats providing diets containing up to 50 ppm of vanadium [45]. Moreover, the dose used in our study is 600 times less than the toxic dose as reported by several workers in rat models [46–48]. Again, we admit that toxicity becomes more relevant only when it comes to human intervention studies, because doing ten thousands of animal experimentations might not be adequate to comment on human toxicity assuming the human exposure levels. In humans, treatment for 6–10 weeks with 4.5–18 mg vanadium/day produced only slight toxicity that too in the form of cramps and diarrhoea [49]. Again, administration of 4.5 mg vanadium/day for 16 months to patients showed no signs of
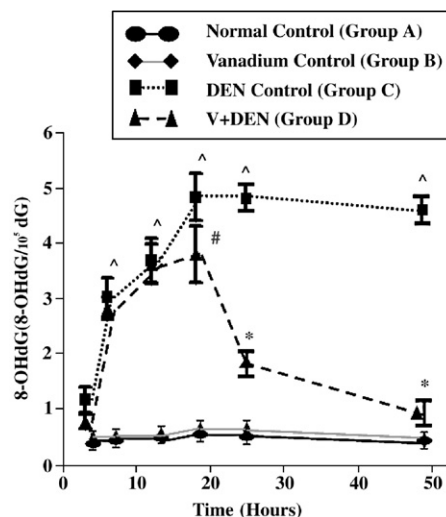

Fig. 5. Levels of formation and persistence of oxidative DNA bases 8-OHdGs in rat liver at sequential time points in presence (Group D) or absence (Group C) of 0.5 ppm vanadium supplementation following a single, necrogenic dose (200 mg/kg body weight) of diethylnitrosamine (DEN) injection. Each bar indicates Mean ± S.E. [n = 15]. ^P < 0.0001 when compared with Normal Control (Group A) at 6, 12, 18, 24 and 48 h of DEN injection; $$P$ < 0.02, #P < 0.001 and *P < 0.0001 respectively at 18, 24 and 48 h after DEN injection when compared with DEN Control (Group C).
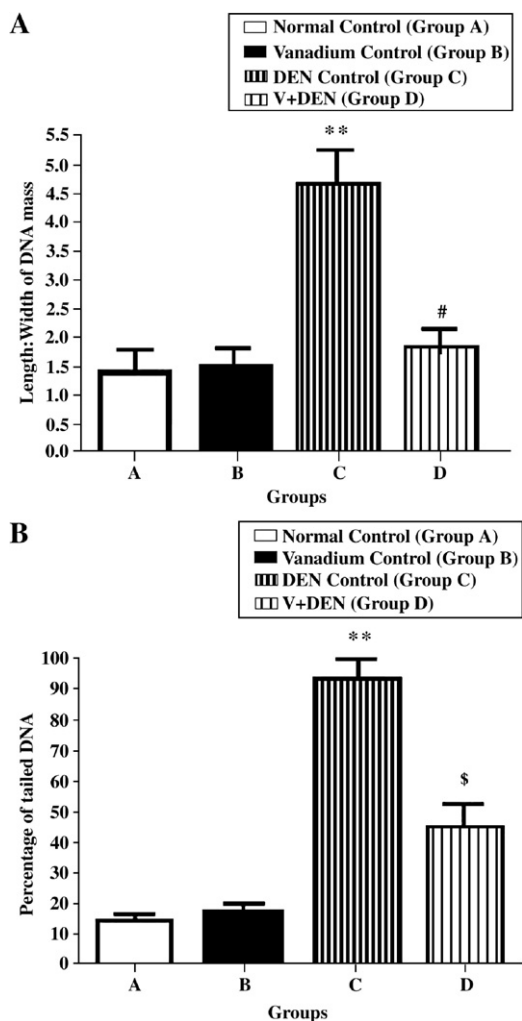
Fig. 6. Effect of 0.5 ppm vanadium on the (A) Length:Width of DNA mass, and (B) Percentage of Tailed DNA in liver of rats 18–20 h after a single, necrogenic dose (200 mg/kg body weight) of diethylnitrosamine (DEN) injection at week 4 of the experimental protocol. Each column and bar indicates Mean±S.E. [$n=15$]. **$P<0.0001$ (Fig. 4A and B) when compared with normal control (Group A); #$P<0.01$ (Fig. 4A) and $P<0.001$ (Fig. 4B) when compared with DEN control (Group C).

supplementary vanadium, as observed in our study may be important for cancer chemoprevention. This could be explained in the light of the fact that, although the precursor lesions were still present in the livers of vanadium treated rats, their growth rates slowed to such an extent that the appearance of visible persistent nodules was delayed beyond the experimental end point owing to an increased latency period [54]. Histopathological impressions further indicate that the hyperplastic nodular hepatocytes formed solid aggregates of one or more cells thick, the prominent "hyperbasophilic focal lesion" mainly around the portal vein in carcinogen-treated rats. The clear and acidophilic cells primarily form AHF, which are considered to be the small "preneoplastic focal lesions" that lead to malignant transformation in later stages of carcinogenesis with the formation of neoplastic nodules and ultimately hepatocellular carcinomas [55]. Thus, the majority of the neoplastic nodules consist of a mixture of preneoplastic, truly neoplastic and diverse intermediate cells. However, long-term vanadium treatment resulted in reduced hepatocyte aggregation and basophilicity with a reversal of heterogeneity towards normal cytology. Histopathological findings as an end-point biomarker primarily confirm the chemopreventive potential of vanadium in inhibiting hepatocellular preneoplastic lesions at the optimum dose (0.5 ppm or 4.27 μmol/L) used in our study. Additionally, dose–response studies of vanadium in relation to morphometric evaluation of preneoplastic lesions and early DNA damage clearly demonstrate the chemopreventive activity of the pharmacologically effective dose of vanadium (0.5 ppm) in vivo. All these, taken together, do justify the chemopreventive role of 0.5 ppm of vanadium in our chemical rat hepatocarcinogenesis model used in this study.

Covalent binding or crosslinking of a reactive carcinogen or its metabolite(s) with the DNA molecule has been postulated to be the primary event in the initiation phase of chemical carcinogenesis. DEN, although a potent genotoxic hepatocarcinogen, yet cannot interact with the DNA molecule as such until and unless it is metabolically oxidized and subsequently decomposes to form the highly reactive alkylating species, namely ethyl diazonium ion ($+N_2C_2H_5$) or ethylcarbonium ($+CH_3CH_2$) ion by the CYP2E1 isozyme in rats and humans [56–58]. Once formed, such electrophilic alkylating agents attack nucleophilic centers in DNA strands (e.g., *N7* and *O6* of guanine moieties) resulting in alkylated/ethylated molecular adducts, such as e[7]Gua and O[6]eGua [59–62]. Replication of such adducted DNA can bring about mutations in the GC base-pairs of activated oncogenes leading to carcinogenesis [56,63]. Using the ^{32}P post-labeling assay, we detected here a substantial level of ethylguanines in hepatic DNA of rats treated with DEN. The maximum level of adducts was formed 18–20 h following DEN threat. This is an indicator of the 'initiation' event of carcinogenesis. After that, the tissue levels of adducts declined in carcinogen challenged liver tissue indicating that, the downstream events of chemical carcinogenesis resume after 18–20 h of DEN threat leading to the development of preneoplastic phenotype ultimately. We have further noticed a substantial decrease in the tissue adduct levels following vanadium

toxicity throughout the study [50]. Other human vanadium trials have also demonstrated a high tolerance for oral vanadium at pharmacological doses greater than 100 times the usual dietary intake [51,52]. Thus, when considering ingestion of up to 125 mg vanadium per day or 8300 mg in 3 months caused only cramps and transient diarrhoea without any other serious toxic manifestations [49], it is evident that 0.5 ppm is really a "low" dose of vanadium used in our experiment. This particular low dose of vanadium results in anticarcinogenicity without any toxicity whatsoever. Therefore, this dose is very much safe and effective pharmacologically.

Even though 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 hepatocellular carcinoma [53]. In view of this, inhibition of nodule incidence and enhancement of their regression by 0.5 ppm of

supplementation. This could be an early potential mechanism for vanadium-mediated inhibition of hepatocarcinogenesis.

Induction of a significant and high steady level of 8-OHdGs essentially plays a critical role for the activation of carcinogenic properties of cells [42,64,65]. Using the sensitive HPLC-ECD method, we detected here a substantial level of 8-OHdGs in hepatic DNA of rats treated with DEN over the vehicle control that supported this concept. The maximum level of modified bases was formed 18–20 h following DEN challenge. This indicates that 8-OHdG may also have a role in the initiation of DEN-carcinogenesis besides ethylguanines. Moreover, the longer maintenance of high levels of 8-OHdGs in liver DNA could be explained by the exhaustion and/or disturbance of DNA repair mechanisms leading to further DNA damages, such as strand-breaks [30,66]. The present investigation showed a prominent suppressive effect of vanadium at 0.5 ppm dose on the levels of 8-OHdGs in DEN-challenged rat liver. Thus, vanadium-mediated reduction of promutagenic lesions might be important in modulating the initiation process of hepatocarcinogenesis.

For the cell, double-strand DNA breaks (DSBs) are the most deleterious form of DNA damage and are generated when the two complementary stands of the DNA double helix are broken simultaneously at sites that are sufficiently close to one another that base-pairing and chromatin structure are insufficient to keep the two DNA ends juxtaposed. As a consequence, the two DNA ends generated by a DSB are liable to become physically dissociated from one another, making ensuing error-prone repair to take place and providing the opportunity for inappropriate recombination with other sites in the genome [67,68]. DSBs are the potent inducers of genomic mutations and of cell death. Depending upon the severity and extent of DSBs damage, cells may either choose to arrest the cell-cycle until the damage is repaired, or if the damage is irreparable, proceed toward apoptosis [67,69]. Error-prone/inaccurate repair or lack of repair of DSBs may lead to mutations or to larger-scale chromosomal aberrations and genomic instability through the generation of dicentric or acentric chromosomal fragments [67]. Such genome changes, including translocations, rearrangements, amplifications, and deletions arising during misrepair may have tumourigenic potential and may contribute to carcinogenesis. Several reports and experimental evidences have shown that, there exists a causal link between the generation of DSBs and the induction of chromosomal mutations with tumourigenic potential [67,68,70–75]. In addition, mutations in many of the factors involved in DSB signaling and repair lead to increased predisposition to cancer in people and in animal models. Indeed, defects in cellular responses to DSBs may be a frequent initiating event of carcinogenesis [67,70,72]. Moreover, formation of DSBs from unrepaired SSBs may lead to progression of preoplastic tissue towards carcinoma. Thus, SSBs may be considered as fundamental to the maintenance of chromosome integrity and genomic stability. The formation of DNA adducts in conjunction with DNA strand-breaks thus indicates a positive correlation between these two molecular events, corroborating a previous report [76]. In the present study, vanadium supplementation resulted in a substantial decrease in the amount of

'tailed' DNA and DNA 'comets' produced by the DEN treatment. This reduction in DNA damage may reflect the potential of vanadium to reduce the genotoxic insult caused by DEN in preneoplastic rat liver.

Conclusively, carcinogen (DEN) insultation to cells may primarily result in the alkylation of hepatic DNA forming tissue-specific ethylated DNA adducts which is a critical step in the initiation of carcinogenesis. DEN-induced alkylation damage of hepatic DNA may furthermore be reflected in inducing DNA lesions, such as DNA strand-breaks in rat liver. Induction of high levels of 8-OHdGs and SSBs may also lead to chromosome breaks and clastogenesis and genotoxic DNA damages are the important manifestations of oxidative stress following carcinogen assault. Oxidative stress has thus been implicated in the initiation of carcinogenesis. The formation of 8-OHdGs is the result of direct interaction between reactive oxygen species (ROS), especially hydroxyl radicals (·OH) and DNA bases [77,78]. Therefore, factors interfering with the generation of ·OH would affect the formation of 8-OHdGs. In this study, it was found that low-dose of vanadium pre-treatment at a concentration of 0.5 ppm in drinking water for 4 weeks significantly reduced 8-OHdG levels in rat liver, indicating a protective effect of vanadium against oxidative DNA damage. Furthermore, in aqueous solution, vanadium is found predominately as oxo-anions (e.g., VO_4^{3-}) and as such may exhibit nucleophilic character for the electrophilic agents to attack, thereby preventing DNA alkylation damage as per the "carcinogen interception mechanism" proposed by Hamilton and Wilker [79–82]. Recently, this group showed that vanadates were able to convert ethylating agents and alkylating toxins into the relatively harmless product ethanol. Their studies suggest that the design of new compounds for cancer prevention should incorporate reactive oxo-groups with high anionic charge density [82]. Thus, the ability of vanadium to attenuate the formation of tissue-specific DNA adducts in liver may indicate its broad-spectrum potential to modulate the kinetics of adduct formation and removal in vivo [81,82]. There is a correlation between inhibition of carcinogenesis by trace metals and lowering of carcinogen binding to cells and DNA. These might involve molecular interactions between metal and carcinogen at different enzymatic and regulatory sites of target cells undergoing neoplastic transformation, as well as stimulation of the host immune system [83]. It is well known that DNA strand-breaks responsible for chromosomal aberrations are generated from DNA base-lesions induced by most of the chemical carcinogens; therefore we may assume that the anticlastogenic effect of vanadium against oxidative DNA lesions in vivo might be at least due to the promotion of excision-repair [84,85]. Again, it has also been established that DSBs are generated from carcinogen-induced DNA lesions in the S phase of the cell cycle. It has been shown that DSBs are repaired by the post-replicational repair in the G_2 phase and that unrepaired DSBs result in chromosomal aberrations [67,70–75]. In this context, we may speculate that vanadium might have a role in the modification of the capability of the post-replicational repair of DSBs. Recent works of Zhang et al. [86] have shown that vanadium is able to induce S and G_2-M phase

cell cycle growth arrest in a dose- and time-dependent manner through activation of p53- and p21-dependent pathways, and may thus prolong the repair mechanisms of oxidative DNA lesions and breakage-type aberrations. However, elucidation of the mechanistic insights into the vanadium-mediated DNA repair pathways remains our future course of study.

Taken together, the study indicates the potential role of vanadium in limiting the initiation event of hepatocarcinogenesis in rats. The study further reflects the interactions of vanadium with the critical molecule DNA, thereby interfering with the formation of DNA adducts and subsequent DNA lesions. This may result in the enhancement of the chemopreventive potential of this trace element in inhibiting the initiation event and thereby limiting genotoxicity during the early stages of hepatocellular transformation in rats. The intricate molecular mechanisms behind the potential anticarcinogenic action of vanadium, particularly in modulating the initiation events warrant a thorough investigation and our present research is focused in this particular direction.

Acknowledgements

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