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Combined Supplementation of Vanadium and Fish Oil Suppresses Tumor Growth, Cell Proliferation and Induces Apoptosis in DMBA-Induced Rat Mammary Carcinogenesis

Sangita Manna, Subhadeep Das, Mary Chatterjee, M. Janarthan, and Malay Chatterjee*

Department of Pharmaceutical Technology, Jadavpur University, P.O. Box - 17028, Kolkata - 700 032, India

ABSTRACT

The anti-cancer activity of vanadium and fish oil has been shown in a large number of studies. This study was undertaken to analyze the combined effect of vanadium and fish oil on 7,12-dimethylbenz(α)anthracene (DMBA)-induced mammary carcinogenesis in female Sprague–Dawley rats. The whole experiment was divided into three parts: (1) DNA strand breaks study, (2) morphological analysis, and (3) histological and immunohistochemical study. Rats were treated with DMBA (0.5 mg/0.2 ml corn oil/100 g body weight) by a tail vein injection. Rats received vanadium (w/v) as ammonium monovanadate at a concentration of 0.5 ppm (4.27 μmol/L) in the drinking water and given *ad libitum* and/or fish oil (0.5 ml/day/rat) by oral gavage. Histology, morphology, DNA strand breaks, cell proliferation, and apoptosis of the mammary tissue were assessed in this study. Treatment with vanadium or fish oil alone significantly reduced the DNA strand breaks, palpable mammary tumors, tumor multiplicity, and cell proliferation but the maximum protection effect was found in the group that received both vanadium and fish oil and the combination treatment offered an additive effect ($P < 0.05$). Furthermore, vanadium and fish oil significantly increased the TUNEL-positive apoptotic cells ($P < 0.05$) but the increase was maximal with combination treatment and had an additive effect. These results affirm the benefits of administration of vanadium and fish oil in the prevention of rat mammary carcinogenesis which was associated with reduced DNA strand breaks, palpable mammary tumors and cell proliferation and increased TUNEL-positive apoptotic cells. J. Cell. Biochem. 112: 2327–2339, 2011.

KEY WORDS: APOPTOSIS; CELL PROLIFERATION; DNA DAMAGE; FISH OIL; VANADIUM

Long-chain polyunsaturated fatty acids (PUFAs) constitute an essential component in human nutrition. PUFAs, particularly those found in fish oils, have been known for their beneficial biological roles in human chronic diseases, in particular, carcinogenesis. There is epidemiological, clinical and experimental evidence that dietary fish oil containing n-3 poly-unsaturated fatty acids (n-3 PUFAs) including eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) protects against the development of breast, colorectal and prostate cancer (Hardman, 2002; Dommels et al., 2003; Kobayashi et al., 2004; Hilakivi-Clarke et al., 2005; Yi et al., 2007; Seti et al., 2009). Vanadium is an important trace element for different organisms. Vanadium is present in oils, fats, fruits, vegetables, cereals, liver, fish, spinach, oysters, shellfish, black pepper, and parsley. Studies from our laboratory have established vanadium as a novel biological regulator in assessing the physiological and biochemical state of animals in a dose related manner and have been shown to inhibit growth and metastasis of breast (Ray et al., 2007), colon (Samanta et al., 2008), and liver (Chakraborty et al., 2007a) cancer cells in vitro and in vivo. 7,12-dimethylbenz(α)anthracene (DMBA) mammary carcinogenesis in rats has been widely used in various mammary cancer chemopreventive studies. This model is preferred, because experimental conditions and requirements are well established and accepted (Russo et al., 1990).

All living organisms are constantly exposed to environmental stresses that cause damage. Carcinogen-induced DNA damage has been implicated one of the early steps in chemical carcinogenesis. Over time and without adequate repair, DNA damage can lead to increased cancer incidence (Halliwell, 2002). Double-strand breaks (DSBs) in cells can be produced by genotoxic agents (ionizing radiation, oxidative damage, chemical agents), often through conversion of single-strand lesions into double-strand breaks during DNA replication in growing cells (Nishino and Morikawa, 2002).

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*Correspondence to: Malay Chatterjee, Department of Pharmaceutical Technology, Jadavpur University, P.O. Box - 17028, Kolkata - 700 032, India. E-mail: mcbiochem@yahoo.com

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The net gain of cells, either through excessive proliferation or through failure of cell death, is a hallmark feature of cancer. Many genes involved in cancer biology directly impact cell proliferation. BrdU is readily incorporated into nuclei during the DNA synthetic phase of the cell cycle (S-phase) and is detected by immunohistochemistry (IHC) with an anti-BrdU antibody. Programmed cell death (PCD) and apoptosis are physiological forms of active cell death that have been associated with specific phases of development. Apoptotic cell death can be clearly visualized morphologically on the basis of cell shrinkage, cell membrane blebbing, chromatin condensation, and nuclear fragments into membrane-enclosed apoptotic bodies. The programmed nature of cell death by apoptosis has suggested that expression of some genes is specifically associated with apoptosis regulation. One of the mechanisms in limiting uncontrolled cell growth has been found to occur through p53-mediated induction of apoptosis in vivo (Okada and Mak, 2004). The p53 tumor suppressor protein plays a pivotal role in the prevention of cellular transformation by curtailing the proliferation of cells harboring potentially oncogenic lesions (Brown et al., 2007). Bcl-2 blocks apoptosis and thereby may contribute to tumorigenesis by prolonging cell survival rather than by accelerating the rate of cell proliferation. Bcl-2 is a cytoplasmic protein belonging to the bcl-2 family, is expressed in normal glandular epithelium, but it is over-expressed in 25–50% of breast cancers (Lee et al., 2007). In the clinical setting, Bcl-2 is up-regulated when apoptosis is suppressed, and an overexpression of Bax leads breast cancer cells to apoptosis in vitro. Bax and Bcl-2 are two Bcl-2 family members that have been well studied for their functions in different apoptosis pathways. Bax expression is significantly reduced or absent in invasive ductal breast carcinoma (Feuerhake et al., 2000). Both gain of Bcl-2 function and loss of Bax function are associated with enhanced survival of breast cancer cells and resistance to apoptosis.

Previous studies from our laboratory have reported that supplementation of vanadium markedly suppressed DEN-induced hepatocarcinogenesis in male Sprague–Dawley rats and DMBA-induced mammary carcinogenesis in female Sprague–Dawley rats by reducing cell proliferation and apoptosis (Chakraborty et al., 2007a; Ray et al., 2007). Similarly fish oil, also, has been found to possess anti-proliferative activities in various cell lines and against certain malignancies in vivo (Dekoj et al., 2007). The present study is the first experiment which has been undertaken to find out the chemopreventive effect of vanadium and fish oil either alone or in combination on DNA strand breaks, morphological analysis, cell proliferation, and apoptosis by modulating the expressions of p53, Bax, and Bcl-2 in 7,12-dimethylbenz(α)anthracene (DMBA)-induced carcinogenesis in female Sprague–Dawley rats.

EXPERIMENTAL METHODS

ANIMALS AND HOUSING

Three weeks old (60–70 g body weights) inbred virgin female Sprague–Dawley (SD) rats, were purchased from Indian Institute of Chemical Biology (IICB), CSIR [Kolkata, India]. Then the animals were acclimatized for 2 weeks. During the acclimatization and experimental period, the animals were housed in Tarson Cages in standard laboratory conditions [humidity 55 ± 5%, lighting (12 h light/12 h dark cycle) and temperature 23 ± 2 °C]. After acclimatization, animals were provided with AIN-76 based diet ((casein – 25 g, DL-methionine – 0.3 g, wheat starch – 35.7 g, cellulose – 6 g, sucrose – 27.4 g, AIN-76 vitamin mixture – 4 g, and AIN-76 mineral mixture – 1.6 g)) and de-mineralized drinking 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.

CHEMICALS

All the reagents and chemicals unless otherwise mentioned were obtained from Sigma Chemicals Co. (St Louis, MO, USA), E. Merck (Frankfurter Straβe, Darmstadt, Germany), Santa Cruz Biotechnology (Santa Cruz, CA, USA) and B D Pharmingen, (San Diego, CA, USA), Upstate Biotechnology (Temecula, CA, USA), Invitrogen (Eugene, OR, USA).

TREATMENT OF ANIMALS

The entire experiment consisted of three parts:

(I) Analysis of DNA-strand breaks in the mammary tissue

After acclimatization of 72 animals, were divided into eight treatment groups of 9 rats each. Groups A, B, C, and D rats were the DMBA treated rats that received a single, tail vein injection of 0.5 mg DMBA/0.2 ml corn oil/100 g body weight at 11 weeks of age. Group A rats were the DMBA control while group a rats were the normal vehicle control that received only single tail vein injection of 0.2 ml corn oil/100 g body weight. Group B and b rats received only vanadium (w/v) as ammonium monovanadate at a concentration of 0.5 ppm (4.27 μmol/L) in the drinking water and given ad libitum starting at 5th week and continued upto 11th week till the administration of DMBA. Group C and c rats received only fish oil by oral gavage at a daily dose of 0.5 ml of Maxepa (Merck, India) [a commercially available preparation of concentrated fish oil rich in omega-3 fatty acids, {a gelatin capsule (1 ml) contains 180 mg EPA and 120 mg DHA}] starting at 5th week and continued up to 11th week till the administration of DMBA. Group D and d received both vanadium and fish oil at the above mentioned doses and time period. For estimation of DNA strand-breaks, rats were killed after 18–20 h after DMBA injection. All the treatments were withdrawn prior to DMBA administration.

DNA ISOLATION AND ASSAY OF STRAND BREAKS

DNA was isolated from the frozen mammary gland of rats of all the groups (Groups A, a, B, b, C, c, D, and d) by a modification of the published procedure of Gupta (Gupta, 1984) with enzymatic RNA digestion before proteinase K treatment of the homogenized tissue. DNA concentration and its purity were estimated spectrophotometrically (Reddy et al., 1984) and then the solution was stored at −20 °C. The DNA unwinding assay was performed according to the procedure of Sarkar et al. (1997). Estimation of SSBs per DNA fragment was done from the procedure used by Basak (Basak, 1996).

(II) Analysis of morphology in the mammary tissue

A total number of 60 rats were divided into four groups (Groups A, B, C, D) (15 rats/group). A total of 15 rats were assigned to the DMBA control group (Group A), another 45 rats (15 rats each group) were assigned to the DMBA + vanadium-treated group (Group B), DMBA + fish oil-treated group (Group C) and DMBA + vanadium + fish oil-treated group (Group D). Vanadium and fish oil were supplemented at the above-mentioned doses and 2 weeks (5 weeks of age) prior to DMBA administration. At 7 weeks of age animals of groups A, B, C, and D were given a single, intra venous tail vein DMBA injection at a dose of 0.5 mg DMBA/0.2 ml corn oil/100 g body weight in female SD rats. Vanadium and fish oil treatments were continued for 36 weeks (41 weeks of animal age). All the treatments were withdrawn a week before the animals were sacrificed.

MORPHOLOGICAL STUDY

Beginning at 4 weeks after DMBA administration, the animals of these four groups were palpated weekly to monitor the presence and location of mammary tumors. The time of appearance of the first tumor (latency period) and the relative size, volume, and location of every tumor were recorded. We have calculated the number of rats with tumors (incidence) and the average number of tumors per tumor-bearing rats (tumor multiplicity) on a weekly basis and at the end of the study. At the termination of the experiment, the two largest perpendicular diameter of each tumor were measured with calipers and the mean of the two measures was used to estimate the tumor size (Noguchi et al., 1997) and the tumor volume was calculated using the formula $V = 4/3\,\pi r^3$ where r is half the average diameter (Harris et al., 2000). Animals of groups A, B, C, and D were sacrificed at 37 weeks (42 weeks of animal age) after DMBA administration under ether anesthesia. The body weights of animals also were recorded twice per week. The animals were palpated weekly to check for the development of palpable mammary tumors and the time of tumor appearance was also recorded.

(III) Analysis of histology, DNA strand breaks, cell proliferation, and apoptosis in the mammary tissue

A total number of 72 rats were divided into eight groups (Fig. 1) (9 rats/group). Vanadium was supplemented at the above mentioned doses throughout the study to the groups B, b, D and d animals, 2 weeks (5 weeks of age) prior to DMBA administration and fish oil supplementation was started at the above mentioned doses to the groups C, c, D and d animals, 2 weeks (5 weeks of age) prior to DMBA administration. At 7 weeks of age animals of groups A, B, C, and D were given a single, intra venous tail vein DMBA injection at a dose of 0.5 mg DMBA/0.2 ml corn oil/100 g body weight in rats. Control rats (Groups a, b, c, and d) were subjected to a similar protocol but received only single tail vein injection of 0.2 ml corn oil/100 g body weight. Vanadium and fish oil treatments were continued for 25 weeks (30 weeks of animal age). All the treatments were withdrawn a week before the animals were sacrificed.

HISTOLOGICAL EVALUATION OF MAMMARY TISSUE

Twenty-four weeks after the carcinogen or vehicle treatment, animals from each group were randomly selected; the thoracic and abdominal inguinal mammary tissues were excised from ether-anesthetized rats, fixed in 10% formalin and processed for histological studies. The tissues were dehydrated through 70, 90, and 100% alcohol and embedded in low melting-point paraffin wax. Sections of 5 μm thickness were cut and placed serially on glass slides. The sections were deparaffinized in xylene and rehydrated through 100, 90, and 70% alcohol. Three contiguous sections were made from each mammary tissue and stained with hematoxylin and eosin for histological evaluation using light microscopy. The histological slides were coded so that the particular sample identity was unknown to the individual making the assessment (Manna et al., 2007).

DNA-STRAND BREAKS ASSAY BY IMMUNOSTAINING WITH γ-H2AX

Immunohistochemical detection of γ-H2AX in cold acetone fixed, paraffin embedded mammary sections was performed by the avidin-biotin-peroxidase-complex method (Jin et al., 2002). Briefly 5 μm thin sections on lysine-coated slides were deparaffinized and rehydrated. For immunolabeling of γ-H2AX antigen retrievals were facilitated by heating the sections in citrate buffer (pH 6.0) for 20 min. Endogenous peroxidase activity was blocked with 1% H_2O_2 in 0.1 M Tris-NaCl (pH-7.6) for 30 min. After incubation in 5% normal goat serum, sections were then incubated overnight at 4°C with the primary antibody, mousemonoclonal IgG anti-phospho-histone H2AX antibody (Upstate Biotechnology), diluted 1:1000 in 3% goat serum. Samples were then incubated with a goat anti-mouse IgG Alexa 488 secondary antibody (Invitrogen) diluted 1:1000 in 3% goat serum incubated for 30 min at 37°C. This was followed by incubation with streptavidin peroxidase (1:100) for 1 h and subsequent chromagen development with AEC-H_2O_2 solution. The sections were then counterstained with Mayer's hematoxylin, dehydrated and mounted and served as positive control. Negative controls were prepared following all the above-mentioned steps omitting the primary antibodies. The percentage of immuno-positive cells was counted under a light microscope. Approximately 200 cells were counted per field, 10 fields were examined per slide and 10 slides were examined per group (Gavrieli et al., 1992).

The average number of immuno-positive foci = (number of immuno-positive cells per field/total number of cells per field) × 100.

CELL PROLIFERATION ASSAY BY IMMUNOSTAINING WITH 5́-BROMO-2-DEOXYURIDINE (BRDU)

At 6 h before sacrifice animals from each group were given i.p. injections of BrdU at a dose of 50 mg/kg body weight. Only the rats in diestrus phase were used as a standard protocol. The diestrus phase was determined by histological examination of vaginal smears. The animals were anesthetized with ether and mammary tissues were dissected out. Immunohistochemical staining was done according to Zhu et al. (1991). Briefly, the deparaffinized tissue sections were exposed to 0.3% hydrogen peroxide in ethanol for 10 min to block endogenous peroxidase and treated with 2N HCl for 1 h and incubated with trypsin 0.1% for 20 min and then with

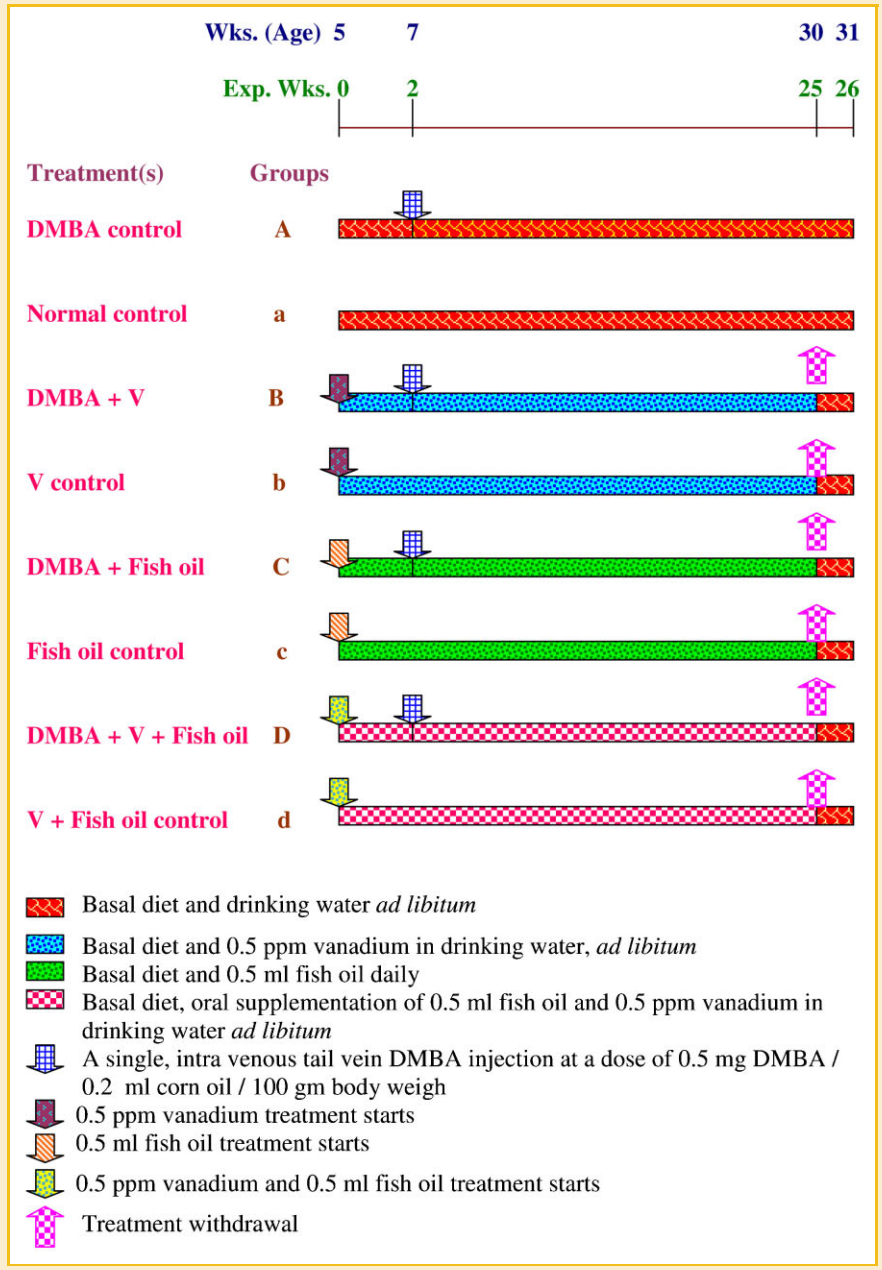

Fig. 1. The basic experimental protocol for histology, DNA strand breaks, cell proliferation, and apoptosis.

normal goat serum for 20 min at room temperature. After tissue sections were incubated with the primary antibody at room temperature (anti-BrdU mouse monoclonal antibody, Sigma) in a humid chamber (1:125 for 2 h) and then with biotinylated anti-mouse IgG (1:200; Sigma) and then with streptavidin horse radish peroxidase which binds to biotin with washing in PBS after each incubation. Visualization was revealed by reaction with 3,3́diaminobenzidine (DAB; Sigma) and 0.04% hydrogen peroxide. All slides were counterstained with Hematoxylin, rinsed, dehydrated and mounted with per mount. Dark brown stains identify the cells incorporating the BrdU-label. Approximately 200 cells were counted per field, 10 fields were examined per slide and 10 slides were examined per group (Gavrieli et al., 1992). The labeling index (LI) was calculated as % of BrdU positive nuclei per total number of cells counted.

The average number of BrdU LI = (number of BrdU-positive cells per field/ total number of cells per field) × 100.

DETECTION OF APOPTOSIS BY TUNEL ASSAY

Terminal dUTP-mediated Nick-End Labeling (TUNEL) assay was performed in formalin-fixed, paraffin embedded tissue sections of mammary gland. Sections were first treated with 20 μg/ml proteinase K in phosphate buffered saline (PBS) for 20 min, quenched by 2% H_2O_2 for 5 min at room temperature and then incubated with terminal deoxynucleotidyl transferase (TdT) buffer (30 mM Trizma base, pH 7.2, 140 mM sodium cacodylate, 1 mM

cobalt chloride), followed by TdT reaction solution containing TdT and dUTP for 60 min at 37 °C. The reaction was stopped with 2% standard saline citrate and incubated with anti-digoxigenin-peroxidase (Sigma) for 30 mins. Color was developed using AEC-H_2O_2 solution (3-amino 9-ethyl carbazole-10 mg, N, N dimethyl formamide—2.5 ml, 0.1N acetate buffer—47.5 ml, 3% H_2O_2—0.5 ml, Sigma) for 4–5 min. The sections were then lightly counterstained with Mayer's hematoxylin and then washed, dehydrated, and mounted. The numbers of cells containing apoptotic nuclei were identified by the development of brown stain. Approximately 200 cells were counted per field, 10 fields were examined per slide and 10 slides were examined per group (Gavrieli et al., 1992).

IMMUNOSTAININGS OF P53, BCL-2 AND BAX

Immunohistochemical detection of p53, Bax and Bcl-2 proteins in cold acetone fixed, paraffin embedded mammary sections was performed by the avidin-biotin-peroxidase-complex method (Jin et al., 2002). Briefly 5 μm thin sections on lysine-coated slides were deparaffinized and rehydrated. For immunolabeling of p53, Bax, and Bcl-2 antigen retrievals were facilitated by heating the sections in citrate buffer (pH 6.0) for 20 min. Endogenous peroxidase activity was blocked with 1% H_2O_2 in 0.1 M Tris-NaCl (pH-7.6) for 30 min. After incubation in 5% normal goat serum, sections were then separately incubated overnight at 4°C with the respective primary antibodies, anti-sheep p53 antibody (Sigma), anti-Bax (N-20) antibody (Santa Cruz Biotechnology) and mouse anti-rat Bcl-2 antibody (Sigma), at 1:200, 1:100, and 1:100 dilutions, respectively in 1% BSA. Sections were then incubated with a biotinylated secondary antibody goat anti rabbit IgG (Sigma) for 30 min at 37°C with 1:100 dilution. This was followed by incubation with streptavidin peroxidase (1:100) for 1 h and subsequent chromagen development with AEC-H_2O_2 solution. The sections were then counterstained with Mayer's hematoxylin, dehydrated and mounted and served as positive control. Negative controls were prepared following all the above-mentioned steps omitting the primary antibodies. The percentage of immuno-positive cells was counted under a light microscope. Approximately 200 cells were counted per field, 10 fields were examined per slide, and 10 slides were examined per group (Gavrieli et al., 1992).

The average number of immuno-positive foci = (number of immuno-positive cells per field/total number of cells per field) × 100.

For each experiment three sets of observation consisting of 15 rats per group for morphological analysis and 9 rats per group for remaining experiments were done. The results indicated as mean of all the three sets that were performed in this study.

STATISTICAL ANALYSIS

General statistical indices like mean and standard deviation were used to describe the features of the data. The significance of differences between control and experimental rats was determined by Student's *t*-test. Tumor incidence was analyzed by Fisher's exact probability test. Additive effects of treatment of vanadium and fish oil were assessed using two-way ANOVA and three-way ANOVA. The results were considered statistically significant if the *P* value was < 0.05.

RESULTS

GENERAL OBSERVATIONS

No significant difference was observed in body weights between the groups (Data not shown). This indicates that vanadium and fish oil supplementations at the particular dose was well tolerated and had no adverse effect on the growth responses of the rats.

MORTALITY

Three rats (20%) from group A (DMBA control), 1 rat (6%) from group B (DMBA + vanadium), 2 rats (13%) from group C (DMBA + fish oil), and 1 rat (6%) from group D (DMBA + vanadium + fish oil) died from the experimental protocol II (Morphological study).

EFFECT OF VANADIUM AND/OR FISH OIL ON DNA STRAND BREAKS ASSAY

From the above study it was observed that a single tail vein injection of DMBA in group A animals resulted in a significant rise in total percentage of DNA single-strand breaks when compared with normal control, i.e., group a. It can be seen that the percentage of native double-stranded DNA of group A animals were found to be 3-fold ($P < 0.05$) less, and the aberrant single-stranded regions in group A animals were 9-fold ($P < 0.05$) higher than in normal control animals (i.e., Group a) (Fig. 2, Table I). This depicted the DNA damaging efficacy of DMBA. In contrast, a statistically significant ($P < 0.05$) decrease in total single- stranded DNA generation was observed in the DMBA + vanadium, DMBA + fish oil and DMBA + vanadium + fish oil-treated animals (Group B, C, and D, respectively). However, maximum protective effect against generation of single-stranded region was found in group D that received both vanadium and fish oil. Moreover the native double stranded DNA in group D animals was almost two-fold higher than in group A animals. There was a significant ($P < 0.05$) increase in the number of

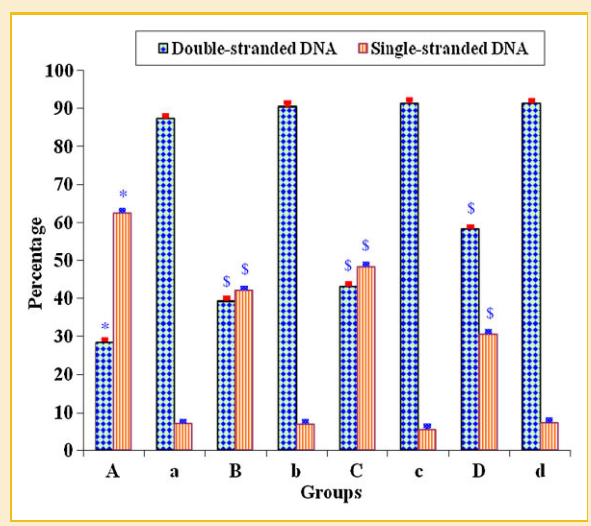

Fig. 2. Effect of vanadium and/or fish oil on the generation of DNA chain breaks in mammary gland of rats in the presence or absence of DMBA treatment. $^{*}P < 0.05$ compared with Group a; $\$P < 0.05$ compared with Group A.

TABLE I. Effect of Vanadium and/or Fish Oil on the Generation of DNA Single-Strand Breaks in Rat Mammary Tissue 18–20 h After a Single Injection of DMBA

Group	Treatment(s)	DS DNA (Mean% ± SD) [n = 9]	SS DNA (Mean% ± SD) [n = 9]	Average no. of SS-Breaks/DNA Unit [n = 9]	Inhibition (%)
A	DMBA control	28.45 ± 0.64*	62.46 ± 0.82*	1.55 ± 0.22*	–
a	Normal control	87.34 ± 0.94	7.17 ± 0.59	0.07 ± 0.02	–
B	DMBA + V	39.23 ± 0.89**	42.19 ± 0.66**	0.65 ± 0.08**	58.06
b	V control	90.54 ± 0.88	6.98 ± 0.61	0.06 ± 0.01	–
C	DMBA + Fish oil	43.27 ± 0.85**	48.35 ± 0.87**	0.68 ± 0.10**	56.12
c	Fish oil control	91.31 ± 0.96	5.53 ± 0.83	0.08 ± 0.01	–
D	DMBA + V + Fish oil	58.28 ± 0.60**	30.57 ± 0.88**	0.51 ± 0.07**	67.09
d	V + fish oil control	91.25 ± 0.85	7.36 ± 0.69	0.04 ± 0.01	–

Results are the Mean ± SD.
*$P < 0.05$ as compared with Normal control (Group a).
**$P < 0.05$ as compared with DMBA control (Group A).
From 2^3 ANOVA analysis, the main effect of A, B, and C and the interaction effect of A-B and A–C are significant ($P < 0.05$). The interaction effect between B and C is not significant ($F = 0.57$), which signifies that the combined effect of vanadium and fish oil was additive in nature. *Source*: A = DMBA; B = vanadium; C = fish oil.

single-strand breaks/DNA could be observed after DMBA injection when compared with normal vehicle control. DMBA-induced animal Treated with vanadium or fish oil (Group B and C respectively) alone abated a decrement at the level of 58.06% and 56.12%, respectively ($P < 0.05$) but the maximum protection effect (67.09%, $P < 0.05$) was found in the group that received both DMBA + vanadium and fish oil (Group D). 2^3 ANOVA (table not shown) analysis of the data showed that the interaction between vanadium and fish oil was statistically insignificant ($F = 0.57$). It signifies that vanadium and fish oil produce an effect that was greater than the individual effect of the former. *Source*: A = DMBA, B = vanadium and C = fish oil.

EFFECT OF VANADIUM AND/OR FISH OIL ON DNA STRAND BREAKS (γ-H2AX ASSAY)

The percentage of γ-H2AX-immuno positive cells in DMBA-induced mammary tissue (Group A) were markedly higher (62.07%) than in the normal control (Group a) as calculated by LI (labeling index) (Table II). The γ-H2AX-LI in the DMBA control group (Group A) was 18.8 ± 0.85 and in DMBA + vanadium (Group B), DMBA + fish oil (Group C) and DMBA + vanadium + fish oil (Group D) were 15.9 ± 0.98, 15.3 ± 0.76 and 14.1 ± 0.94, respectively. Thus, there were substantial reduction in γ-H2AX-LI in vanadium-treated group (Group B) by 15.43%, ($P < 0.05$), in fish oil treated group (Group C) by 18.62%, ($P < 0.05$) and in vanadium + fish oil-treated group (group D) by 25%, ($P < 0.05$) when compared to DMBA control group (Group A). The γ-H2AX-labeled cells showed a distinct nuclear localization (Figures not shown). However, the most beneficial effect could be observed in group D rats when the vanadium and fish oil combination was used; an additive inhibitory effect was produced the interaction effect between them is statistically not significant ($F = 0.73$) (ANOVA 2^3 analysis), which signifies that the effect of the vanadium and fish oil was additive in nature. Rats in groups a, b, c, and d (Control groups of A, B, C, and D, respectively) did not show any detectable γ-H2AX-immunopositive cell.

TABLE II. γ-H2AX-LI in Mammary Tissue

Groups	γ-H2AX-LI
Group A (DMBA control)	18.8 ± 0.85*
Group a (Normal control)	11.6 ± 0.88
Group B (DMBA + vanadium)	15.9 ± 0.98**
Group b (Vanadium control)	11.1 ± 0.89
Group C (DMBA + fish oil)	15.3 ± 0.76**
Group c (Fish oil control)	11.8 ± 0.72
Group D (DMBA + vanadium + fish oil	14.1 ± 0.94**
Group d (Vanadium + fish oil control)	11.6 ± 0.67

LI = labeling index.
γ-H2AX-LI = percentage of γ-H2AX-labeled cell/total number of cells counted.
Results are the Mean ± SD.
*$P < 0.05$ as compared with Normal control (Group a).
**$P < 0.05$ as compared with DMBA control (Group A).
From 2^3 ANOVA analysis, the main effect of A, B and C and the interaction effect of A-B and A-C are significant ($P < 0.05$). The interaction effect between B and C is not significant ($F = 0.73$), which signifies that the combined effect of vanadium and fish oil was additive in nature. *Source*: A = DMBA; B = vanadium; C = fish oil.

MORPHOLOGICAL FINDINGS

The incidence (%) of palpable mammary tumors in the DMBA control (Group A), DMBA + vanadium-treated group (Group B), DMBA + fish oil-treated group (Group C) and DMBA + vanadium + fish oil-treated group (Group D) is shown in Table III. In the DMBA control group (Group A), 91.6% of animals had tumors with an average of 8.09 tumors per tumor-bearing rat (tumor multiplicity) at the end of 35 weeks (42 weeks of animal age). The mean latency period of tumor appearance was as early as 12 weeks post-carcinogen treatment. The mammary tumor usually appeared one at a time with additional tumors appearing even several weeks after the first tumor was observed. The majority of tumors appeared between 15 and 25 weeks and multiple tumors of different sizes were frequently found in the same animal. Most of the tumors were between 2 and 5 mm in size. When vanadium and fish oil alone or in combination were given to the carcinogen-treated animals (groups B, C, and D, respectively), its inhibitory action could be ascertained from the reduced tumor incidence. In these groups only 64.3, 69.2, and 50%, respectively of rats had tumors at the end of 35 weeks (42 weeks of animal age), which are significantly different ($P < 0.05$) from group A. The mean latency period for tumor appearance for these groups were almost 17 weeks, 16 weeks, and 20 weeks,

TABLE III. Effect of Vanadium and/or Fish Oil (Maxepa) on the Incidence, Growth and Development of DMBA-Induced Palpable Mammary Tumors in Rats After 35 Weeks

Group	Latency period (weeks)	Total no of tumors	No of rats with tumors per total rats	No of rats with tumors (incidence %)	No of tumors/rat (tumor multiplicity)*	Tumor size (mm)		Tumor volume (mm^3)**
						<2	>2–<5	
Group A	12	89	11/12	91.6	8.09 ± 0.68	34	55	1.5 ± 0.03
Group B	17	38	9/14	64.3***	4.22 ± 0.85***	14	24	0.8 ± 0.01***
Group C	16	44	9/13	69.2***	4.88 ± 0.58***	16	28	0.9 ± 0.01***
Group D	20	26	7/14	50***	3.71 ± 0.43***	9	17	0.6 ± 0.07***

Group A = DMBA control; Group B = DMBA + vanadium, Group C = DMBA + fish oil, Group D = DMBA + V + fish oil
*Mean number of tumors/animal and SD.
**Mean tumor volume and SD.
***Significantly different from group A by *t*-test ($P < 0.05$).
From 2^2 ANOVA analysis, the interaction effect between A and B is not significant ($F = 1.01$), which signifies that the combined effect of vanadium and fish oil is additive in nature. *Source*: A = DMBA + vanadium; B = DMBA + fish oil.

respectively post-carcinogen treatment, which are significantly ($P < 0.05$) longer than group A. The number of tumors per tumor-bearing rat (tumor multiplicity) was also significantly ($P < 0.05$) reduced showing a 47.83, 39.67, and 54.14%, respectively decrease in tumor multiplicity (Table III). There were a decrease in the percentage of rats developing tumors with time in the vanadium and fish oil alone or in combination treated groups. Vanadium and fish oil treatments also characteristically attenuated the number of tumors of 2 and 5 mm in size and also volume of tumors indicating slower tumor progression. The 2^2 ANOVA (table not shown) analysis of tumor multiplicity depicted that vanadium and fish oil when supplemented together had an additive effect in inhibiting the tumor multiplicity, because the interaction effects between them is not significant ($F = 1.01$). *Source*: A = DMBA + vanadium, B = DMBA + fish oil.

MAMMARY HISTOLOGY

Sections from normal control rat mammary tissue (Group a) showed preserved normal ductular and alveolar structure of mammary tissue with epithelial cells of uniform appearance (Fig. 3a). Mammary tissue from DMBA-treated group (Group A) showed hyperplasia of the lobules characterized by a typical arborization and formation of clusters of cells that lined the dialated ducts of the alveolus (Fig. 3A). The cellular architecture was found to be altered and enlargement of the alveolus was seen with cells showing nuclear pleomorphism, characterized by nuclear enlargement, clumping of chromatids, and prominent nucleoli. Atypical epithelial hyperplasia was observed. Epithelial cells showed variation in nuclear size with irregular chromatin and prominent nucleoli. However, the myoepithelial layer was preserved in the alveolar structure and it could not be considered as frank carcinoma at this stage. It was observed from the histological slides that the vanadium and fish oil alone or in combination-treated DMBA group (Groups B, C, and D, respectively) presented a histological profile almost similar to the normal control [Figs. 3 (B, C, and D, respectively)]. In these groups, there were mild ductular proliferations with focal epithelial hyperplasia. Epithelial cells were uniform in size and anisonucleosis and mitosis were absent. Periductular adipose tissue was present with patchy inflammatory cellular infiltrate (composed mainly of lymphocytes). The vanadium control, fish oil control and vanadium + fish oil control (Groups b, c and d) showed no observable distinct change from the normal control (Figures not shown).

EFFECT OF VANADIUM AND/OR FISH OIL ON CELL PROLIFERATION (BRDU)

To analyze the chemopreventive action of vanadium, fish oil or both in combinations, its effect on cellular proliferation in the mammary gland were examined. The percentage of BrdU-labeled cells as calculated by LI (labeling index) was more in the carcinogen control group A (Table IV). The BrdU-LI in the DMBA control group (Group A) was 25.9 ± 0.92 and in DMBA + vanadium (Group B), DMBA + fish oil (Group C), and DMBA + vanadium + fish oil (Group D) were 19.6 ± 0.72, 19.8 ± 0.62, and 17.7 ± 0.44, respectively. Thus, there were substantial reduction in BrdU-LI in vanadium-treated group (Group B) by 24.32%, ($P < 0.05$), in fish oil treated group (Group C) by 23.55%, ($P < 0.05$) and in vanadium + fish oil-treated group (group D) by 31.66%, ($P < 0.05$) when compared to DMBA control group (Group A). The BrdU-labeled cells showed a distinct nuclear localization (Figures not shown). Moreover, 2^3 ANOVA analysis of the BrdU-positive cell proliferation data showed that the interaction effect between B and C is not significant ($F = 0.57$), which signifies that the combined effect of vanadium and fish oil was additive in nature. *Source*: A = DMBA; B = vanadium; C = fish oil.

EFFECT OF VANADIUM AND/OR FISH OIL ON APOPTOSIS

The TUNEL positive apoptotic cells in DMBA-induced mammary tissue (Group A) (Fig. 4A) were markedly lower (65.38%) than in the normal control (Group a). DMBA-induced rats treated with vanadium and fish oil alone or in combination (Groups B, C, and D) produced a significant ($P < 0.05$) increase in apoptotic index in the mammary tissue as compared to the DMBA control rats (Group A). In the Groups B, C, and D, which received either vanadium or fish oil or both vanadium and fish oil, respectively; the expression of TUNEL positive apoptotic index increased to a substantial level but the most prominent induction was found in group D rats. Vanadium and fish oil alone (Groups B and C, respectively) increased the number of apoptotic index by 55.55% ($P < 0.05$) and 51.85% ($P < 0.05$), respectively, whereas the combined (Group D) supple-

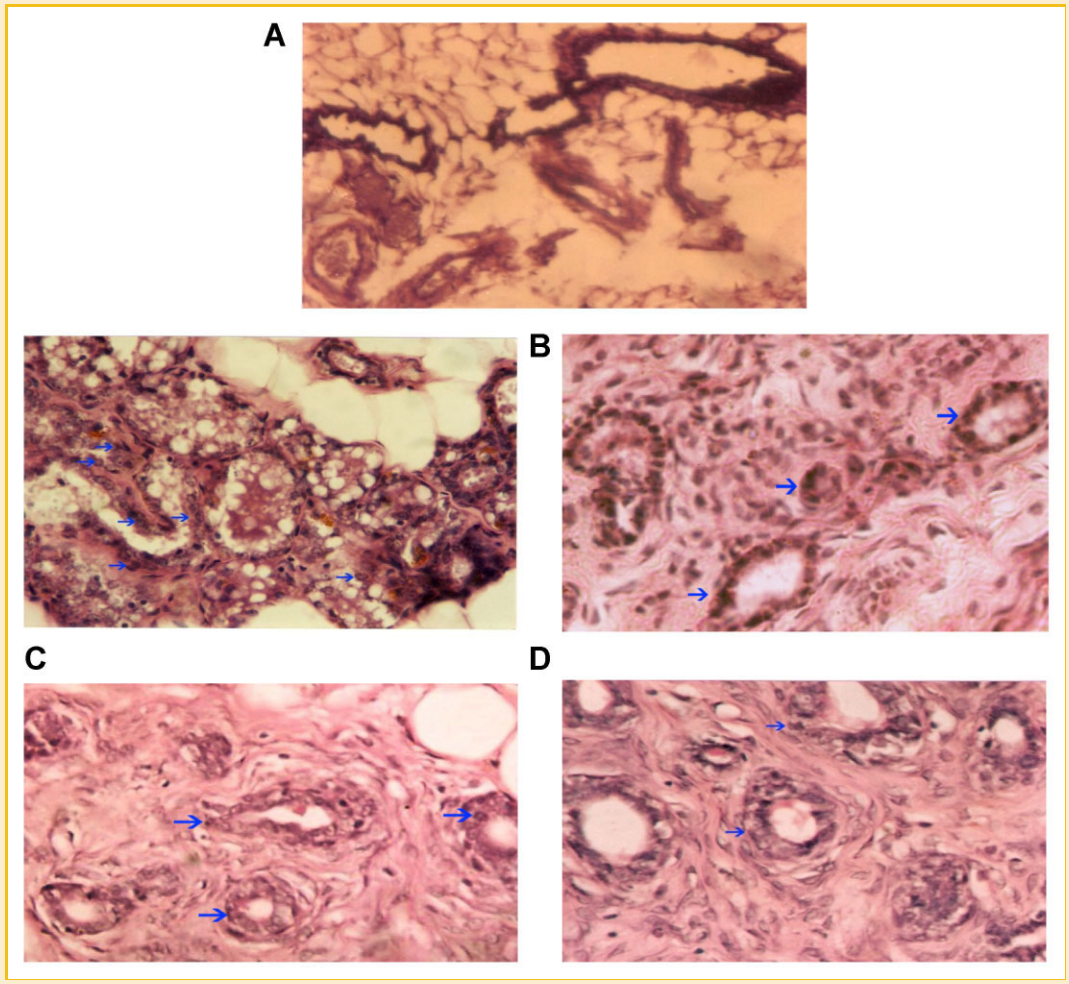

Fig. 3. Histological sections of mammary tissue of rats showing (a) normal cellular architecture (normal control, group a); (A) marked proliferation of ductal epithelial lining with hyperchromatic enlarged nuclei (DMBA control, group A) (marked with arrows [→]); and (B, C and D) mild ductular proliferations with focal epithelial hyperplasia, uniform in size (DMBA + vanadium, group B; DMBA + fish oil, group C, and DMBA + vanadium + fish oil, group D) (marked with arrows). Magnification, H & E ×25.

mentation of both over the same time period exerted maximum induction of expression of apoptotic index by 85.18% ($P < 0.05$) when compared to DMBA control group (Group A). However, the most beneficial effect could be observed in group D rats when the vanadium and fish oil combination was used; an additive inhibitory effect was produced the interaction effect between them is statistically not significant ($F = 0.73$) (ANOVA 2^3 analysis), which signifies that the effect of the vanadium and fish oil was additive in nature. Rats in groups a, b, c, and d (Control groups of A, B, C, and D, respectively) did not show any detectable apoptotic index. Figures 4A, 4B, 4C, and 4D show the TUNEL positive apoptotic tissues of Groups A, B, C, and D rats, respectively. *Source*: A = DMBA, B = vanadium and C = fish oil.

TABLE IV. BrdU-LI in Mammary Tissue

Groups	BrdU-LI
Group A (DMBA control)	$25.9 \pm 0.92^{*}$
Group a (Normal control)	11.3 ± 0.95
Group B (DMBA + vanadium)	$19.6 \pm 0.72^{**}$
Group b (Vanadium control)	11.6 ± 0.72
Group C (DMBA + fish oil)	$19.8 \pm 0.62^{**}$
Group c (Fish oil control)	11.4 ± 0.82
Group D (DMBA + vanadium + fish oil)	$17.7 \pm 0.44^{**}$
Group d (Vanadium + fish oil control)	11.8 ± 0.53

LI = labeling index.
BrdU-LI = percentage of BrdU-labeled cell/total number of cells counted.
Results are the Mean ± SD.
$^{*}P < 0.05$ as compared with Normal control (Group a).
$^{**}P < 0.05$ as compared with DMBA control (Group A).
From 2^3 ANOVA analysis, the main effect of A, B and C and the interaction effect of A-B and A–C are significant ($P < 0.05$). The interaction effect between B and C is not significant ($F = 0.57$), which signifies that the combined effect of vanadium and fish oil was additive in nature. *Source*: A = DMBA; B = vanadium; C = fish oil.

EFFECT OF VANADIUM AND/OR FISH OIL ON P53, BAX, BCL-2 EXPRESSIONS

A few p53 immuno-positive cells with a mean of 2.55 ± 0.15 were detected in the mammary tissue sections of the carcinogen control group (Group A) animals. Treatment with vanadium or fish oil or both in combination in groups B, C, and D rats, respectively showed an increase ($P < 0.05$) in p53 immuno-reactivity with a mean of 3.94 ± 0.18, 3.86 ± 0.34, and 4.48 ± 0.19, respectively, when compared to carcinogen control group (Table V). Thus, there were substantial inductions in p53 immuno-positive cells in vanadium-

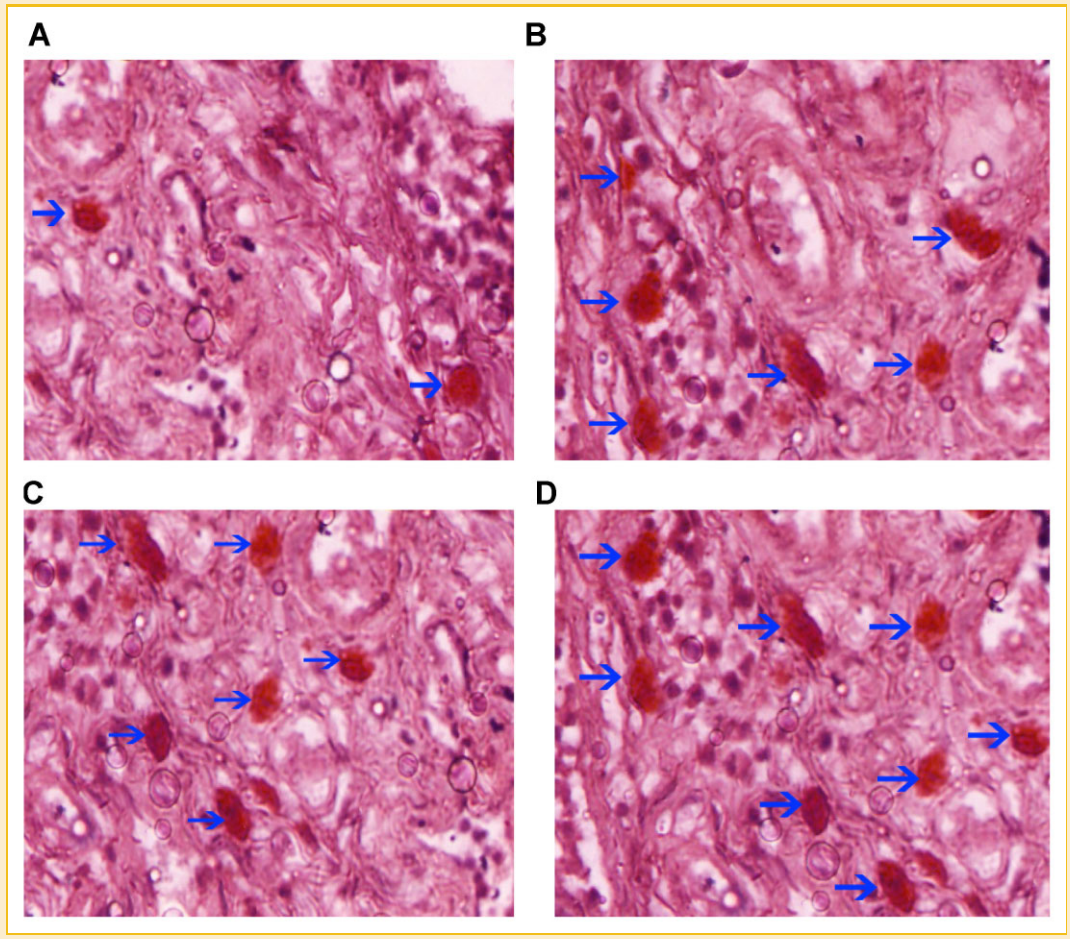

Fig. 4. Representative immunohistochemistry photomicrographs of TUNEL-positive apoptotic cells in mammary tissue. Arrows (→) indicate TUNEL-positive cells in mammary tissue of rats. (A) DMBA control group (Group A); (B) DMBA + vanadium-treated group (Group B); (C) DMBA + fish oil-treated group (Group C), (D) DMBA + vanadium + fish oil-treated group (Group D). Magnification ×25.

treated group (Group B) by 54.51%, ($P < 0.05$), in fish oil-treated group (Group C) by 51.37%, ($P < 0.05$), and in vanadium + fish oil-treated group (Group D) by 75.69%, ($P < 0.05$) when compared to DMBA control group (Group A). Moreover, 2^3 ANOVA analysis of the data showed that, the maximum induction of p53 immunoreactivity was found in group D rats that received both vanadium and fish oil (Figure not shown), they produced an additive inhibitory effect (the interaction effect between vanadium and fish oil is

TABLE V. p53-, Bax-and Bcl-2-Immunopositive Cells of the DMBA-Induced Mammary Tissue After a 25 weeks Vanadium and Fish Oil Either Alone or in Combined Treatment

	p53-immuno positive cells*	Bax-immuno positive cells*	Bcl-2-immuno positive cells*
Treatment group	Mean ± SD	Mean ± SD	Mean ± SD
A (DMBA control)	$2.55 \pm 0.15^{**}$	$3.74 \pm 0.27^{**}$	$9.32 \pm 0.36^{**}$
a (Normal control)	6.34 ± 0.23	8.44 ± 0.18	3.76 ± 0.19
B (DMBA + V)	$3.94 \pm 0.18^{***}$	$5.66 \pm 0.32^{***}$	$6.02 \pm 0.26^{***}$
b (V control)	6.76 ± 0.28	8.32 ± 0.37	3.26 ± 0.43
C (DMBA + fish oil)	$3.86 \pm 0.34^{***}$	$5.47 \pm 0.43^{***}$	$6.53 \pm 0.47^{***}$
c (Fish oil control)	6.18 ± 0.31	8.65 ± 0.49	3.23 ± 0.38
D (DMBA + V + fish oil)	$4.48 \pm 0.19^{***}$	$6.26 \pm 0.38^{***}$	$5.46 \pm 0.27^{***}$
d (V + fish oil control)	6.63 ± 0.29	8.72 ± 0.25	3.43 ± 0.24

*Results are the Mean ± SD. Results were analyzed by 2^3 ANOVA.
**$P < 0.05$ as compared with Normal control (Group a).
***$P < 0.05$ as compared with DMBA control (Group A).
From 2^3 ANOVA analysis, the main effect of A, B and C and the interaction effect of A-B and A-C are significant ($P < 0.05$). The interaction effect between B and C is not significant (($F = 1.12$ for p53; $F = 0.32$ for Bax and $F = 0.74$ for Bcl-2)), which signifies that the combined effect of vanadium and fish oil was additive in nature. *Source*: A = DMBA; B = vanadium; C = fish oil.

statistically not significant ($F = 1.12$), which signifies that the combined effect of vanadium and fish oil was additive in nature. *Source*: A = DMBA; B = vanadium; C = fish oil.

A few Bax immuno-positive cells (3.74 ± 0.27) were detected in the mammary tissue sections of the carcinogen control group (Group A) whereas increased in Bax immuno-positivity (5.66 ± 0.32, 5.47 ± 0.43, and 6.26 ± 0.38, respectively, $P < 0.05$) were found upon vanadium (Group B), fish oil (Group C), and vanadium + fish oil (Group D) supplementations (Table V). Thus, there were substantial inductions in Bax immuno-positive cells in vanadium-treated group (Group B) by 51.34%, ($P < 0.05$), in fish oil-treated group (Group C) by 46.26%, ($P < 0.05$) and in vanadium + fish oil-treated group (Group D) by 67.38%, ($P < 0.05$) when compared to DMBA control group (Group A) (Figure not shown). ANOVA analysis of the data showed that, maximum beneficial effect was produced, when the vanadium and fish oil combination was used, because the interaction effect between them is statistically not significant ($F = 0.32$), which signifies that the combined effect of vanadium and fish oil was additive in nature. *Source*: A = DMBA; B = vanadium; C = fish oil.

Bcl-2 protein was detected immunohistochemically in the DMBA control group (Group A) as well as in the treatment groups (Group B, C, and D). Overall, Bcl-2 expressions in the treatment with vanadium or fish oil or both in combination in groups B, C, and D rats, respectively, was lower with a mean of 6.02 ± 0.26, 6.53 ± 0.47, and 5.46 ± 0.27 when compared to the DMBA control group (Group A) where the expression was much higher with a mean of 9.32 ± 0.36 ($P < 0.05$) (Table V). Thus, there were substantial reductions in Bcl-2 immuno-positive cells in vanadium-treated group (Group B) by 35.41%, ($P < 0.05$), in fish oil-treated group (Group C) by 29.94%, ($P < 0.05$) and in vanadium + fish oil-treated group (Group D) by 41.42%, ($P < 0.05$) when compared to DMBA control group (Group A) (Figure not shown). It was observed from the ANOVA 2^3 analysis data (table not shown) that when vanadium and fish oil combination was used, they produced an additive inhibitory effect because the interaction between them is statistically insignificant ($F = 0.74$). *Source*: A = DMBA; B = vanadium; C = fish oil.

DISCUSSION

The efficacy of combination therapy with various anticancer agents has now been generally recognized. Our results showed that dietary exposure to vanadium and fish oil reduced DNA strand breaks with reduction of the expression of γ-H2AX in pre-malignant lesions in mammary tissue of rats. This experiment suggested that the apparent combined chemopreventive effect of vanadium and fish oil was underscored by the reversal almost to normalcy of the DMBA-induced mammary hyperplasia. The results of the present study clearly demonstrated that combined supplementation of vanadium and fish oil effectively suppressed cell proliferation with concomitant down-regulation of the expression of BrdU, proliferation nuclear antigen and increased apoptosis in a defined Sprague–Dawley rat mammary carcinogenesis model. There was significant increased in p53 and Bax expressions in the mammary tissue of the rats treated simultaneously with DMBA + vanadium, DMBA + fish oil and DMBA + vanadium + fish oil than from the rats treated with DMBA only. Vanadium and fish oil were further effective in decreased Bcl-2 expression in vivo.

We have noted here that concomitant administration of vanadium and fish oil (combined) were significantly reduced ($P < 0.05$) DMBA induced DNA strand breaks when compared to DMBA control rats. Again, when given together they exert an additive effect decreasing the DNA strand breaks. We have also noted that carcinogen induced animals treated with vanadium and/or fish oil reduced the expression of γ-H2AX when compared to only carcinogen-induced animals. But the maximum down-regulation of the expression of γ-H2AX was found in animals when supplemented with both vanadium and fish oil upon compared with DMBA-treated animals. Studies by Rhodes et al. have shown the protective effect of dietary EPA on oxidative DNA strand breaks in peripheral blood lymphocytes against acute UVR-induced genotoxicity; longer-term supplementation might reduced skin cancer in humans (Rhodes et al., 2003). Fish oil supplementation does appears to protect against oxidative stress-induced DNA damage of rat liver and suggested that lipid peroxidation does not enhance but lowers the DNA damage (Kikugawa et al., 2003). Series of studies from our laboratory have established the potential role of vanadium in limiting preneoplastic lesions in liver, colon, and mammary carcinogenesis models in rats (Kanna et al., 2004; Ray et al., 2005; Chakraborty et al., 2007b). We may say evidences were accumulating that the DNA alteration is a contributing factor to the pathogenesis in cancer initiation and other stages of carcinogenesis (Srivastava et al., 2001). DMBA-DNA adducts are depurinating adducts formed by one-electron oxidation, in which the 12- methyl group of DMBA reacts with the N-7 of adenine or guanine respectively (Chakravarti et al., 1995). The formation of DMBA-DNA binary complex in the DNA backbone induces conformational change in DNA leading to a significant increase in the level of DNA nick formation and enhancement of DNA strand breaks after DMBA administration (Daniel et al., 1985). The substantial decrement of the single-strand breaks can explain one possible mechanism of the anti-clastogenic potential of vanadium and fish oil. Many mechanisms seem to be operational in n-3 fatty acid mediated chemoprevention in vivo. The protective effect of fish oil against oxidative DNA damage may be regulated to lower arachidonic acid, COX-2 and PGE_2 induced inflammation or else enhancing apoptosis (Hong et al., 2005). Vanadium may stabilize xenobiotic enzymes, thereby minimizing the formation and binding of carcinogen-derived free radicals with cellular macromolecules and thus prevents DNA lesions and clastogenesis.

An observation of a significant percentage of rats with tumors after vanadium and fish oil treatment can be explained by assuming the carcinogenic effect, even if initiated, was suppressed to a considerable extent as indicated by a long latency period of tumor development. The potential chemopreventive role of vanadium and fish oil was also reflected in the reduced number of tumors per tumor-bearing rat. But an additive inhibitory effect was found in the group D rats that received both vanadium and fish oil.

Fish oil supplements along with vanadium significantly reduced ($P < 0.005$) the numbers of the BrdU positive foci in Group D rats which received both vanadium and fish oil as compared to the other

treatment groups (Group B and C which received vanadium and fish oil, respectively). Previous studies from our laboratory have suggested that supplementation of vanadium markedly suppressed DEN-induced hepatocarcinogenesis in male Sprague-Dawley rats and DMBA-induced mammary carcinogenesis in female Sprague–Dawley rats by reducing BrdU cell proliferation (Ray et al., 2006; Chakraborty et al., 2007a). Long-term feeding of both olive and fish oil diets modulates azoxymethane-induced colon carcinogenesis, decreased BrdU incorporation and the expression of cytosolic beta-catenin and cyclin D and increased apoptosis in the colon mucosa (Fujise et al., 2007). Low-dose eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) inhibited cell kinetics as evidenced by a decreased bromodeoxyuridine-labeling index, of a 7,12-dimethyl-benz(a)anthracene-induced mammary carcinoma in rats (Minami & Noguchi, 1996). It was found by Albright et al. that conjugated linoleic acid decreased proliferation in MCF7 cells, as measured by the incorporation of bromodeoxyuridine (BrdU) (Albright et al., 2005). From above results, we confirmed that vanadium and fish oil are anti-proliferating agents in mammary cancer therapy. The involvement of fish oil (Maxepa) in suppressing neoplastic transformation of mammary tissue might be mediated through the decreased of cell proliferation (Manna et al., 2007; Manna et al., 2010). The mechanism of the anticarcinogenic effect of vanadium may involve inhibition of metabolic activation of the pro-carcinogen, leading to reduced generation and/or binding of the ultimate carcinogen to DNA (Chatterjee and Bishayee, 1998). Regardless of the mechanism, based on the results reported here, vanadium and fish oil in combination trigger a unique protective effect against the expression of BrdU positive foci by a potent carcinogen DMBA.

In this study a significant up-regulation of apoptotic index in chemically-induced rat mammary carcinogenesis model following supplementation of vanadium and fish oil was observed. An additive inhibitory effect was found in the group D rats that received both vanadium and fish oil. Vanadium has been reported to exert its antitumor effects by inhibiting cellular tyrosine phosphatase and/or activation of tyrosine phosphocyclases (Evangelou, 2002). In animal studies, highly purified n-3 PUFA ethyl esters have been shown to be incorporated into colonocyte mitochondrial membrane phospholipids, which coincided with the enhancement of apoptosis in the colon (Jin et al., 2002). Fish oil has been reported to induce apoptosis in a number of cancer cell lines, including breast carcinoma cell lines (Chiu et al., 2004). This increased in mammary epithelial cell apoptosis after vanadium and fish oil treatments, coupled with significant reduction in tumorigenesis may create an environment in the mammary gland that is less susceptible to chemical carcinogenesis.

Several studies have suggested that p53 status is an important determinant of tumor responsiveness to anti-neoplastic agents (Wilson et al., 1997). Studies of Sharma et al. indicate that under the influence of omega-3 fatty acids, in ovarian cancer epithelial cell lines, there are definitive growth suppressive mechanisms at work and that the biologic effects of omega-3 fatty acids may in part be mediated by the wild-type p53 status (Sharma et al., 2005). We have observed here, vanadium and fish oil supplementations elevated the level of p53, suggesting thereby that, vanadium + fish oil-mediated induction of apoptosis might be at least due to wild type p53 expression in vivo and this combined supplementations are also additive in nature. However, detailed studies are needed to confirm the role of a p53-dependent pathway in vanadium and fish oil-mediated apoptosis in mammary cancer. The process of programmed cell death or apoptosis is regulated by apoptosis-related genes or regulatory molecules. The major apoptotic pathway signal transduction cascades associated with PCD include the proteins of Bcl-2 family. In this experiment, a significant decrease in Bcl-2 expression as observed in stages of mammary preneoplasia during vanadium and fish oil supplementation suggested that, down regulation of Bcl-2 might be one of the possibilities by which vanadium and fish oil may sensitize cancer cells to apoptosis but it warrants further study. This is noteworthy that dietary fish oil creates an environment permissive for apoptosis, thereby reducing cancer risk (Sanders et al., 2004). n-3 PUFA has been shown to decreased (anti apoptotic) Bcl-2 expression in colon cancer cell lines (Avivi-Green et al., 2002). Sheng et al. reported that prostaglandin E_2 (PGE_2) inhibited apoptosis via induction of Bcl-2 expression. PGE_2 is produced by cyclooxygenase (COX)-I and II from arachidonic acid and is responsible for inflammation injury to cells leading to carcinogenesis (Sheng et al., 1998). Dietary fish oil was found to decrease both PGE_2 and COX-II levels (Singh et al., 1997). Some of the members of the Bcl-2 family which are over-expressed in a variety of human cancers (e.g., Bcl-2, Bcl-xl) are blockers of cell death, while others, such as Bax and Bcl-xs, are promoters of apoptosis, and their levels might be reduced in some types of cancers (Sarkar et al., 2003). Our results are compatible with those studies in that there is a significant decreased in the expression of Bcl-2 and an increased in the expression of Bax in the preneoplastic mammary tissue of rats treated with vanadium and fish oil. Group B and C rats those received vanadium and fish oil alone, respectively, showed a decreased level of Bcl-2 and increased level of Bax but an additive effect was found in vanadium plus fish oil-treated rats (Group D). Previous study from our laboratory has shown that vanadium induced apoptosis by induction of proapoptotic gene Bax (Ray et al., 2007). Both conjugated linoleic acid, which contains conjugated double bonds, and EPA (CEPA) have anti-tumor effects. CEPA treatment caused upregulation of expression of genes induced by p53 and activation of the mitochondrial apoptosis pathway via Bax and the death pathway via TRAIL, leading to apoptosis of DLD-1 colorectal adenocarcinoma human cells (Tsuzuki et al., 2007). Again Cheng et al. suggested that increased intake of n-3 PUFAs promoted apoptosis of normal colon mucosa in human which is related to effect on Bax or the balance of Bax and Bcl-2 (Cheng et al., 2003). Our study suggested that the possible induction of apoptosis may occur through vanadium and fish oil. Thus p53, Bax and Bcl-2 act in concert in inducing the possible and there by to limit the growth of the tumor cells in vivo upon dietary supplementation of vanadium and fish oil. The probable mechanism of the vanadium and fish oil mediated inhibition of DMBA-induced carcinogenesis may be that they act as blocking agents in the detoxification of genobiotic compounds which can inhibit the further adduct formation (Manson et al., 2000). These combinations is also effective in reducing the frequency of occurrence of DMBA induced DNA strand breaks predicting its role as suppressing agent as well. All these could reduce the

consequences of altered gene expressions by reducing proliferation of initiated cells and restoring apoptosis in normal level. The observation is thus interesting and warrant further investigation.

CONCLUSION

In conclusion, our study suggested that vanadium and fish oil supplementations reduced DNA strand breaks, cell proliferation, and induced apoptosis and limit growth of tumor cells that involve p53, Bax, and Bcl mediated pathways. But an additive inhibitory effect was found in group D rats which received both vanadium and fish oil. This attribute could be considered important, as this trace element and the fish oil in combination may open new perspective for the human breast preneoplasia in near future.

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