EVALUATION OF COMPARATIVE AND COMBINED ANTIMUTAGENIC POTENTIAL OF VITAMIN C AND VITAMIN E USING HISTIDINE MUTANT *SALMONELLA TYPHIMURIUM* STRAINS

T A Ajith, Marry Ann* and Joe Thomas**

Departments of Biochemistry and **Community Medicine, Amala Institute of Medical Sciences, Amala Nagar, Thrissur, Kerala, India-680 555.

*Department of Biochemistry, Amala Cancer Research Centre, Amala Nagar, Thrissur, Kerala, India-680 555.

ABSTRACT

Chemoprevention represents a new intervention strategy to control some type of carcinogenesis especially in subjects at high risk for cancer development. Experimental and epidemiological data indicate that a variety of nutritional factors including vitamin C and E are effective to lower the risk of some types of cancer. However large prospective studies have failed to find such significant association. A comparative and combined in vitro antimutagenic potential of two antioxidant vitamins ascorbic acid (vitamin C) and α -tocopherol (vitamin E) were evaluated using Ames's Salmonella typhimurium test assay. Directly acting mutagens such as sodium azide (NaN_3) and 4-Nitro-o-phenylenediamine (NPDA), and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) were used to induce mutation in salmonella strains TA 98 and TA 100. Vitamin C significantly ($P < 0.01$) and dose dependently inhibited the mutagenicity induced by all the three mutagens. The percent inhibitions of vitamin C at 15 mg/plate were 33.8% (NaN_3), 52.5 % (MNNG) and 55.4 % (NPDA). Vitamin E (15 mg/plate) was effective to inhibit mutagenicity induced by NaN_3 and MNNG but did not inhibit mutation induced by NPDA. Combination of vitamins (vitamin C plus vitamin E) produced only an additive antimutagenic activity when compared to their activity at 5 mg/plate. The results of the study concluded that vitamin C is a better antimutagenic agent than vitamin E and combination of vitamins did not produce any synergistic activity.

KEY WORDS

Antimutagenicity, Antioxidant, Cancer chemoprevention, Vitamins.

INTRODUCTION

Many chemical and physical carcinogens can induce one or more variety of mutations in cells when given chronically. Reactive oxygen species (ROS) play a major role as endogenous initiators and promoters of DNA damage and in mutations that contribute to cancer, diabetes, heart disease and other age related diseases (1,2). ROS have been conventionally regarded as having carcinogenic potential and have been associated with tumor promotion (3). However

certain ROS have been shown to act as essential second messengers for several cytokines and growth factors (4). The extensive DNA modifications produced by ROS include modified purine and pyrimidine bases e.g. 5-hydroxymethyl uracil (HMU), thymine glycol (TG) or 8-hydroxyguanine (8-OHG) (5). Oxidants have the capacity to induce the transcription of growth competence related protooncogene *C-fos* and *C-jun* in several systems. Induction of these immediate genes represent a prerequisite for the stimulation of cell proliferation (6). Accumulation of genetic changes has been found in the development of cancer. Mutations of different origins have been identified in various types of cancers (7). About 50% of human cancers have a point mutation in *K-ras*, *APC* (Adenomatous Polyposis Coli) or *p53* protooncogene (8-10). Mutations of *K-ras* and *APC* can occur in early lesions, while alterations of *p53* and *DCC* often occur in advanced tumors (11).

Address for Correspondence :

Dr. T.A. Ajith,

Department of Biochemistry,

Amala Institute of Medical Sciences,

Amala Nagar, Thrissur, Kerala-680 555, India.

E-mail : taajith@rediffmail.com

In recent years there is an upsurge in the areas of research related to newer developments in the prevention of free radical caused diseases using antioxidants. A large number of studies have shown that increased consumption of fresh fruits and vegetables is associated with a reduced risk for most types of cancer (12). Vitamin C and vitamin E are essential nutrients that function as non-enzymatic antioxidants to prevent the propagation of free radical reactions in the cytosol and cell membrane respectively. Chemoprevention represents a new intervention strategy to control some types of carcinogenesis by using specific natural or synthetic chemical agents that may enhance intrinsic physiological mechanism to protect the organism against the development and progression of mutant clones of malignant cells (13). Modern oncology is being challenged to broaden its focus to chemoprevention of cancer using agents with multiple biological properties. Compounds with antioxidant and antimutagenic activities may provide a promising step towards the selection of successful cancer chemopreventive agents. Antioxidant vitamins, vitamin C and E, had been reported to reduce cancer risk by 20-30% by inactivating the action of free radicals and preventing carcinogen formation from inactive precursors (14). However the results obtained from numerous trials on the preventive role of these vitamins are contradictory and far from conclusive. A combination of different vitamins or of vitamins with other agents may be particularly useful in the cancer treatment. Though the antimutagenic activity of vitamin C and E were reported against oxidant mutagens, a comparative study to select a better one or a study to evaluate their combined synergistic effect has not yet been established. Therefore the current study was aimed to demonstrate the antimutagenic activity of vitamin C and E alone and their combination using *Salmonella typhimurium* tester strains TA98 and TA 100.

MATERIALS AND METHODS

Salmonella typhimurium strains TA98 and TA100 were originally obtained from Prof. B.N. Ames, University of California, Berkeley, USA. The strains were subcultured in nutrient broth for over night and stored at -70°C as frozen permanents in the presence of 9 % dimethyl sulphoxide (DMSO). Thawed frozen permanent (40 ml) was used to inoculate for the fresh overnight culture of each strain in nutrient broth (5 ml). The inoculated nutrient broth was incubated overnight at 37°C and used for the antimutagenicity assay.

Ascorbic acid (vitamin C), DMSO, sodium chloride (NaCl), dextrose (C₆H₁₂O₆), potassium chloride (KCl), ammonium sulphate ((NH₄)₂ SO₄), potassium dihydrogen phosphate (KH₂PO₄), and dipotassium hydrogen phosphate (K₂HPO₄)

were purchased from E. Merck, India Ltd. D-biotin, L-histidine and agar-agar were purchased from Sisco Research Laboratories Pvt. Ltd. Mumbai, India. 4-nitro-*o*-phenylenediamine (NPDA) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) were purchased from Sigma chemical company, St. Louis, USA. Sodium azide (NaN₃) was from Himedia and vitamin E (Evion 400 of Merk Limited) was purchased from Amala Cancer Hospital Pharmacy. All other chemicals and reagents used were of analytical reagent grade.

NaN₃ (2.5 mg/plate, in sterile distilled water), MNNG (1 mg/plate in DMSO), and NPDA (20 mg/plate in DMSO) were used as the directly acting mutagens.

Antimutagenic activity was determined by the method of Maron and Ames (1983) (15). Freshly grown overnight culture of *Salmonella* tester strain (TA 98 and TA100) in nutrient broth (0.1 ml, approximately 10⁹ bacterial cells/ml) was mixed with various concentrations of vitamin C (5, 10, and 15 mg/plate in sterile distilled water) or vitamin E (5,10, and 15 mg/plate in DMSO), 0.2 ml of 0.5 mM histidine/biotin solution and 0.01 or 0.02 ml of mutagen in 2 ml of molten top agar at 45°C. The mixture was poured onto minimal glucose agar plate and incubated for 48 h at 37°C. After the incubation period, number of bacterial revertants per plate were counted using a colony counter. To evaluate the toxicity of the vitamins, plates without mutagens, but with vitamin (at highest concentration tested for antimutagenicity) treated in the similar manner was employed.

All the experiments were repeated twice in triplicate. The percent inhibition of mutagenicity was calculated using the formula $\{(1-(R_2-SR)/(R_1-SR)) \times 100$, where, R₁ is the average number of revertants in the presence of mutagen alone, R₂ is average number of revertants in the presence of mutagen plus vitamin and SR (spontaneous revertants) is the average number of revertants in the plate without vitamins or mutagen. The antimutagenicity will be evident if there is an increased percent inhibition of NaN₃ and NPDA induced mutations when treated with vitamins.

All experimental data were expressed as Mean $\pm$ SD. One-way Analysis of Variance (ANOVA) followed by Dunnett's *t*-test, (using GraphPad Instat software) was applied for detecting the significant difference between mutagen alone and mutagen plus vitamins treated plates. *P*<0.05 was considered as significant.

RESULTS AND DISCUSSION

Vitamin C inhibited NaN_3 and MNNG induced mutation of TA100 and NPDA induced mutation of TA98 in a dose dependent manner (Table 1,2 and 3). However vitamin E could inhibit MNNG and NaN_3 induced mutation to TA 100 only. The percentage inhibition of mutagenicity in the vitamin C (15 mg/plate) + NaN_3 , vitamin C + MNNG and vitamin C + NPDA treated plates were 33.8, 52.5, and 55.4 % respectively. The activity at this concentration was found to be significant ($P<0.01$) and highest. The highest activity of vitamin E at 15 mg/plate against MNNG and NaN_3 induced mutations were 18.3 ($p<0.01$) and 14.4 % ($p<0.05$) respectively. However vitamin E up to the dose of 15 mg/plate did not inhibit the NPDA induced mutations to TA98. Vitamins in combination (5 mg each of vitamin C and E) produced 21.8 and 29.8 % inhibitions of mutations induced by NaN_3 and MNNG respectively to TA 100 (Table 1 and 2). The percent inhibition of NPDA induced mutation to TA98 by vitamins in combination was 21.2%. Results of the present study revealed that the antimutagenic effect of vitamin C was better than that of vitamin E. The effect was in a dose dependent manner. Among the antimutagenic activity of vitamin C, the activity against NPDA induced mutation was found to be the highest. Similarly the antimutagenic effect of vitamin C against MNNG induced mutation to TA100 was also found to be high.

Table 1: Antimutagenic effect of vitamin C, vitamin E and vitamin C+ vitamin E against NaN_3 induced mutation to salmonella strain TA 100

Treatments (mg/plate)	Average number of revertants/plate	Percent inhibition
NaN_3 (0.0025)	1190.0 $\pm$ 82.0	—
NaN_3 + vitamin C (5mg)	1071.3 $\pm$ 55.2 ^{ns}	10.8
NaN_3 + vitamin C (10mg)	984.1 $\pm$ 33.9 **	17.3
NaN_3 + vitamin C (15mg)	787.0 $\pm$ 108.9 **	33.8
NaN_3 + vitamin E (5mg)	1069.0 $\pm$ 69.2 ^{ns}	10.1
NaN_3 + vitamin E (10mg)	1024.5 $\pm$ 22.6 *	13.9
NaN_3 + vitamin E (15mg)	1019.3 $\pm$ 12.7 *	14.4
NaN_3 + vitamin C (5mg) + vitamin E (5mg)	930.0 $\pm$ 16.3**	21.8

Values are Mean $\pm$ SD, n=3,

** $P<0.01$ and * $P<0.05$ (Dunnett's *t*-test) significantly and ^{ns} non-significantly differ from mutagen alone treated group. Average number of revertants/plate expressed after deducting the spontaneous revertants of TA 100 (92.0 $\pm$ 5.6). Average number of revertants/plate of TA 100 in the vitamin C or vitamin E alone treated plates were 100.0 $\pm$ 20.2 and 95.0 $\pm$ 14.0 respectively.

Table 2 : Antimutagenic effect of vitamin C, vitamin E and vitamin C+ vitamin E against MNNG induced mutation to salmonella strain TA 100

Treatments (mg/plate)	Average number of revertants/plate	Percent inhibition
MNNG (0.001)	1294.0 $\pm$ 25.4	—
MNNG + vitamin C (5mg)	1011.5 $\pm$ 15.2**	21.8
MNNG + vitamin C (10mg)	907.0 $\pm$ 49.0**	29.9
MNNG + vitamin C (15mg)	614.0 $\pm$ 76.3**	52.5
MNNG + vitamin E (5mg)	1104.0 $\pm$ 79.1**	14.6
MNNG + vitamin E (10mg)	1068.5 $\pm$ 13.4**	17.5
MNNG + vitamin E (15mg)	1057.0 $\pm$ 74.9**	18.3
MNNG + vitamin C (5mg) + vitamin E (5mg)	908.0 $\pm$ 20.0**	29.8

Values are Mean $\pm$ SD, n=3,

** $P<0.01$ (Dunnett's *t*-test) significantly differ from mutagen alone treated group. Average number of revertants/plate expressed after deducting the spontaneous revertants of TA 100 (92.0 $\pm$ 5.6). Average number of revertants/plate of TA 100 in the vitamin C (15 mg/plate) or vitamin E (15 mg/plate) alone treated plate were 100.0 $\pm$ 20.2 and 95.0 $\pm$ 14.0 respectively.

The antimutagenic activity of vitamin C or E at 5 mg/plate was only moderate in all the experiments. Hence this intermediate dose was selected to evaluate their combined effect. Combination of vitamins, vitamin C plus vitamin E, treatment showed that the combined concentration of these vitamins produced only a sum of their individual antimutagenic activity when compared to the activity of each vitamin at 5 mg/plate. Thus the combination of vitamin C and vitamin E treatment did not produce any synergistic antimutagenic activity. Mutagens that cause base-pair substitution mutations primarily at one of the GC pairs can revert the mutated *S. typhimurium* TA 100 strain to the wild type. In contrast to TA 100, TA 98 is a frame shift mutagen sensitive strain (16). The results of this study demonstrated that vitamin C is effective against both types of mutagens. The antimutagenic activity of these vitamins against directly acting mutagens may probably be due to the protection of bacterial genome from the binding of the mutagens either by eliminating the mutagen from the bacteria or by inhibiting the entry of mutagen to the bacteria. Vitamin E had been reported to inhibit methylazoxy methanol induced mutagenesis in *Salmonella typhimurium* strain TA100 by direct action on the mutagen (17). MNNG induces a wide spectrum of mutations by alkylating purines. The major adduct reported was *O*⁶-methylguanine (18). The antimutagenicity of these vitamins may be due to the inhibition of the MNNG induced alkylation of purine bases. The free radicals may be involved in the NaN_3 induced mutation of the *Salmonella* test strains.

Table 3 : Antimutagenic effect of vitamin C, vitamin E and vitamin C+ vitamin E against NPDA induced mutation to salmonella strain TA 98

Treatments (mg/plate)	Average number of revertants/plate	Percent inhibition
NPDA (0.020)	1310.0 ± 42.4	—
NPDA + vitamin C (5mg)	1086.1 ± 76.3*	17.0
NPDA + vitamin C (10mg)	716.0 ± 94.8**	45.3
NPDA + vitamin C (15mg)	583.0 ± 53.7**	55.4
NPDA + vitamin E (5mg)	1300.0 ± 89.1	—
NPDA + vitamin E (10mg)	1368.5 ± 71.1	—
NPDA + vitamin E (15mg)	1357.0 ± 91.2	—
NPDA + vitamin C (5mg) + vitamin E (5mg)	1031.5 ± 16.3*	21.2

Values are Mean ± SD, n=3,

** $P < 0.01$ and * $P < 0.05$ (Dunnett's *t*-test) significantly differ from mutagen alone treated group. Average number of revertants/plate expressed after deducting the spontaneous revertants of TA 98 (48.5 ± 3.6). Average number of revertants/plate of TA 98 in the vitamin C (15 mg/plate) or vitamin E (15 mg/plate) alone plate were 40.0 ± 10.0 and 44.2 ± 9.0 respectively.

Hence the antioxidant activity of these vitamins could also explain the mechanism of antimutagenicity. Christen et al., demonstrated the antimutagenic activity of gamma-tocopherol by trapping the mutagenic electrophiles (19). Moreover, antioxidant was reported to be effective to inhibit the mutation induced by MNNG, NPDA and NaN_3 to *Salmonella typhimurium* strains (20). The exhibited antimutagenic activities of the vitamins are not due to their toxic effect on the bacterial colonies. This was evident from the vitamin (15 mg/plate) and bacteria treated plates, the background lawn was clear and the number of revertants of bacterial colonies were similar to the bacteria alone treated plate (SR).

Reactive oxygen species (ROS) formed from endogenous or exogenous sources are highly reactive, toxic and mutagenic (21). Many types of cancer are thought to result from oxidative damage to DNA caused by free radicals. Antioxidants possess a variety of biological activities including the induction of drug metabolizing enzymes, inhibition of prostaglandin synthesis, inhibition of carcinogen induced mutagenesis and scavenging of free radicals (22). Cell culture studies indicate that the vitamin E ester, alpha-tocopheryl succinate, can inhibit proliferation and induce apoptosis in a number of cancer cell lines (23,24). However, several large prospective studies have failed to find significant associations between alpha-tocopherol or ascorbic acid supplementation and the incidence of cancers. While research is underway to determine whether

combinations of antioxidant vitamins might be beneficial as an adjunct to conventional cancer therapy, definitive conclusions are not yet possible. The results of numerous trials on the preventive role of several vitamins have been conducted in high-risk population that may not be applicable to other population groups (25). Nevertheless the effect of antioxidant vitamins supplementation in the treatment of cancer, the results of this study concluded that vitamin C is a better antimutagenic agent and combination of vitamins did not produce any synergistic antimutagenic activity.

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