Dietary supplementation of vitamin A, C and E prevents p-dimethylaminoazobenzene induced hepatic DNA damage in rats

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The preventive effect of antioxidant vitamins A, C, E and their analogues against DNA damage induced by a hepatocarcinogen p-dimethylaminoazobenzene (DAB) was assessed by comet assay. For genotoxicity (DNA damage) study, male albino rats were divided into 11 groups, consisting of four rats each. Group I served as control. Group II to VII received 1, 10, 100, 200, 300 and 400 mg per kg body wt of DAB respectively; group VIII to XI received 500 mg/kg body wt of DAB. They were sacrificed by cervical decapitation 3, 6, 12 and 24 h after treatment; livers were excised immediately and subjected to comet assay to measure DNA damage. To study the effect of vitamins, experiments were conducted on a group of 275 rats divided into 3 sets of 25 rats each. First set served as control; second set received 0.06% DAB and third set received 0.06% DAB, along with analogues of vitamins A, C and E. Rats fed with 0.06% DAB were provided water ad libitum for a period of 4 months, followed by a normal (basal) diet for further 2 months. Vitamins A (10,000-50,000 IU), C (75-1000 mg) and E (50-500 mg) and their analogues were given (per kg body wt) to the third set of rats by gavage route once in a week for a period of 6 months. The DAB induced DNA damage only at the highest tested dose of 500 mg/kg body wt. Administration of high doses of vitamin A acid, L-ascorbic acid and vit. E succinate individually prevented the DNA damage. However, administration of a mixture of these vitamins at low doses prevented the DAB-induced DNA damage, which may be due to their synergistic effect. The results indicate that there is a significant advantage in mixed vitamins therapy at low dose over the treatment with individual vitamins.

Keywords: Genotoxicity, p-Dimethylaminoazobenzene, Hepatic DNA damage, Comet assay, Analogues of vitamin A, C and E

Cancer is a major health problem worldwide and one of the most important causes of morbidity and mortality in children and adults. p-Dimethylaminoazobenzene (DAB), a colouring agent commonly used in food stuffs is one of the powerful liver carcinogens. When administered orally, DAB induces hepatocellular carcinoma in rats and even when administered as a single intraperitoneal dose in male mice. The ‘International Agency for Research on Cancer’ has assessed that DAB is possibly carcinogenic (group 2 B) to humans. Several reports indicate that aminoazodyes cause oxidative DNA damage, which in turn lead to carcinogenesis. High-fiber and low-fat diets, fresh fruits and vegetables provide a protective effect against cancer. Fresh fruits and vegetables are a rich source of antioxidant vitamins like A, C and E and hence prevent cellular damage. The scavenging capacity of these vitamins arrests oxidative damage by neutralizing the free radicals that are believed to trigger tumour development and growth. Hence, interest in the use of vitamins as an antioxidant nutrient in cancer therapy is gaining momentum. The present study has been carried out to find out the preventive effect of vitamins A, C and E and their analogues against DAB-induced DNA damage.

The comet assay is used to detect DNA damage induced immediately after the administration of relatively high doses of the carcinogen, while carcinogenicity assay is used for long treatment with low doses. Thus, comet assay is performed to detect the DNA damage, induced shortly after the administration of DAB by gavage route and to study the preventive effect of vitamins. The DAB-induced DNA damage is also assessed, after the administration of vitamins (gavage route) for a period of 6 months.

Material and Methods

Male albino rats (Wistar strain), reared and maintained under the supervision of Institutional Animal Ethics Committee in the animal house of the Department of Biochemistry, J. J. College of Arts and Science, Pudukkottai, were used for the study.

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Genotoxicity study (DNA damage)

Male albino rats (n = 44) weighing 170 ± 20 g each were divided into eleven groups, consisting of 4 rats each. Group I served as control. Each animal of the groups II to VII received a single dose of 1, 10, 100, 200, 300 and 400 mg/kg body wt of DAB respectively, whereas those of the groups VIII to XI received 500 mg/kg body wt of DAB. Subsequently, rats were sacrificed by cervical decapitation after 3, 6, 12 and 24 h and the livers were excised immediately to detect the DNA damage.

Individual vitamin treatment

A group of 275 rats (170 ± 20 g) were divided into 3 sets. First set (n = 25) served as control. The second set (n = 25) received 0.06% of DAB based on the earlier study. The third set consisting of the remaining 225 rats was further subdivided into subsets A, C and E (n = 75) based on the vitamins used. The subsets A, C and E were further divided into sub-subsets of A1, A2 and A3 and C1, C2 and C3 and E1, E2 and E3 respectively consisting 25 rats each. Finally, all the sub-subsets were further divided into 5 groups (Groups I to V) of 5 rats each.

All the rats were acclimatized to laboratory conditions for one week and fed ad libitum with a basal diet (containing 660 g glucose, 180 g milk casein, 40 g salt mixture, 180 g sugar, 200 g corn oil, 20 g cod liver oil, 1.5 g choline chloride, 50 g vitamin K3, 20 mg riboflavin, 20 mg thiamine, 20 mg pyridoxine, 60 mg calcium pantothenate, 50 mg nicotinamide, 1.8 mg folic acid, 0.6 mg biotin, 100 mg inositol, 50 mg p-aminobenzoate and 40 mg cyanocobalamin per kg of diet). Animals of second and third sets were fed with 0.06% DAB supplemented diet. Control rats continued to fed on the basal diet for 6 months. The third set was administered with chosen doses of vitamins (per kg body wt) based on previous study, once in a week by gavage route {A1: DAB + vit. A acetate (10,000-50,000 IU), A2: DAB + vit. A acid (10,000-50,000 IU), A3: DAB + vit. A palmitate (10,000-50,000 IU); C1: DAB + l-ascorbic acid (75-1000 mg), C2: DAB + vitamin C (75-1000 mg), C3: DAB + ascorbyl palmitate (75-1000 mg) and E1: DAB + vit. E (50-500 mg), E2: DAB + vit. E acetate (50-500 mg) and E3: DAB + vit. E succinate (50-500 mg) for a period of 4 months. After this period, the vitamin enriched basal diet was offered for an additional period of 2 months. After 6 months, the rats were sacrificed with ether to excise the liver to assess the DNA damage.

Multiple vitamin treatment

Multiple vitamin treatment was performed with mixture of vit. A, L-ascorbic acid and vit. E succinate. For this study, a set of 35 rats were divided into 7 groups consisting of 5 rats each for normal, DAB alone, DAB + vit. A (10,000 IU) + vit. C (75 mg) + vit. E (50 mg), DAB + vit. A (20,000 IU) + vit. C (150 mg) + vit. E (100 mg), DAB + vit. A (30,000 IU) + vit. C (250 mg) + vit. E (200 mg), DAB + vit. A (40,000 IU) + vit. C (500 mg) + vit. E (400 mg) and DAB + vit. A (50,000 IU) + vit. C (1000 mg) + vit. E (500 mg). The mixture of vitamins was given to all the rats by gavage route once in a week for a period of 6 months.

Comet assay

Comet assay was carried out with slight modifications. The excised livers were minced using mincing solution containing HBSS with 20 mM EDTA and 10% DMSO (pH 7.5). To obtain nuclei, the homogenate was centrifuged at 700 × g for 10 min at 4°C. Slides prepared from isolated nuclei were placed in chilled lysing solution (2.5 M NaCl, 100 mM EDTA, 20 mM Trizma base, pH adjusted to 10 using NaOH pellets, 1% Triton X-100 and 10% DMSO, added immediately before use) for 60 min at 0°C to remove cellular proteins. Thereafter, slides were placed in a gel electrophoresis unit and incubated in fresh alkaline electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH 13) for 40 min at room temperature.

Electrophoresis was performed at 0°C in the dark for 15 min at 25V (300 mA); then the slides were immersed in neutralization buffer (0.4 M Tris–HCl, pH 7.5) and gently washed three-times for 5 min at 4°C to remove alkali and detergents. Finally, the gels stained with ethidium bromide were covered with a cover slip and the slides were examined and photographed at 20× objective (100 nuclei per slide) on an epifluorescence microscope (Nikon, Eclipse E400, Japan). The captured images were scored using Komet 6.0 to measure the percentage of DNA in the comet tail. One hundred randomly selected non-overlapping cells were visually assigned a score on an arbitrary scale of 0-4 (0 = no DNA damage, 1 = mild damage, 2 = moderate damage, 3 = less severe damage and 4 = severe damage, Fig. 1), based on perceived comet tail length migration and relative proportion of DNA in the comet tail.

To determine whether visual scoring was correlated with computerized image analysis, the same cells...
were also scored for DNA damage using Komet 6.0 software. The migration of DNA away from the nucleus, i.e., comet tail length and percentage of DNA in tail were measured. Finally, the tail moment was calculated as follows:

\[
\text{Tail moment} = \frac{\text{Tail length} \times \% \text{ DNA in tail}}{100}
\]

**Statistical analysis**

Statistical analysis of the data was performed using student’s ‘t’ test to determine significant differences between control and treatment groups. Linear regression analysis was applied to correlate the visual comet scores with computerized image analysis derived scores.

**Results**

Fig. 1 shows the comet images with increasing degree of DNA damage of rat liver corresponding to different cell classes of \( p \)-DAB administered (500 mg/kg body wt) rats. No DNA damage of liver was observed, when DAB was administered in the range of 1-400 mg/kg body wt, when compared with control. However, administration of 500 mg DAB/kg body wt resulted in a significant \((P<0.001)\) DNA damage.

**Comparison of visual scoring with Komet 6.0**

A significant correlation \((r = 1.00, P<0.001)\) was found between visual scores of DNA damage and percentage of tail DNA (Fig. 2).

**DNA migration**

The migration of nuclear DNA from rat liver treated with DAB is given in Table 1. None of the samples collected after 3 h of oral administration with 1, 10, 100, 200, 300 and 400 mg of DAB/kg body wt exhibited an increase in DNA migration, when alkaline electrophoresis was conducted at \( \text{pH} > 13 \). However, oral administration of 500 mg/kg body wt resulted in increased migration of liver nuclei after 3, 6, 12 and 24 h.

![Fig. 1—Comet images with increasing degree of DNA damage from rat liver corresponding to cell classes 0-4 [0, no damage; 1, mild damage; 2, moderate damage; 3, less severe damage; and 4, severe damage]](image1.png)

![Fig. 2—Relationship between percent of tail DNA measured using Komet 6.0 software and visual scores of DNA damage from rat liver corresponding to cell classes 0-4 [0, no damage; 1, mild damage; 2, moderate damage; 3, less severe damage; and 4, severe damage. Values are mean ± SD; r, 1, P<0.001]](image2.png)

<table>
<thead>
<tr>
<th>DAB (mg/kg body wt)</th>
<th>Sampling time (h)</th>
<th>Migration (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (Normal rats)</td>
<td>0</td>
<td>1.95 ± 0.10</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td>1.77 ± 0.05</td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>1.96 ± 0.10</td>
</tr>
<tr>
<td>100</td>
<td>3</td>
<td>2.46 ± 0.11</td>
</tr>
<tr>
<td>200</td>
<td>3</td>
<td>2.73 ± 0.14</td>
</tr>
<tr>
<td>300</td>
<td>3</td>
<td>2.76 ± 0.14</td>
</tr>
<tr>
<td>400</td>
<td>3</td>
<td>2.78 ± 0.07</td>
</tr>
<tr>
<td>500</td>
<td>3</td>
<td>14.62 ± 0.36*</td>
</tr>
<tr>
<td>500</td>
<td>6</td>
<td>15.17 ± 0.31*</td>
</tr>
<tr>
<td>500</td>
<td>12</td>
<td>21.0 ± 0.55*</td>
</tr>
<tr>
<td>500</td>
<td>24</td>
<td>23.45 ± 0.38*</td>
</tr>
</tbody>
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*\( P<0.001 \) vs Normal rats

Note: For genotoxicity studies, DAB dissolved in olive oil was given to rats by gavage route with a volume of 10 ml/kg body wt.
### Effect of vitamins on migration of DNA

The effect of vitamins and their analogues on the migration of nuclear DNA from liver of rats treated with DAB is given in Table 2. Vit. A acetate and palmitate supplementation had no significant effect ($P<0.001$) on the migration of nuclear DNA of rats administered with DAB. Vit. A acid at low dose (10,000 IU) could neither arrest the migration of nuclear DNA nor protect the DNA damage; however, at high doses (20,000-50,000 IU), it protected the liver DNA damage. Administration of sodium ascorbate and ascorbyl palmitate had no significant effect ($P<0.001$) on the migration of nuclear DNA. L-Ascorbic acid also protected the liver cells only at high doses (above 250 mg/kg body wt) and was ineffective at low doses. It significantly reduced ($P<0.001$) DAB-induced DNA damage in the liver. Vitamin E succinate had a potential role in protecting the liver DNA from damage. No significant effect was observed on supplementation with vit. E and its acetates. However, vit. E succinate significantly ($P<0.001$) reduced the DNA damage at high doses of 400 and 500, but not at 50, 100 and 200 mg/kg body wt.

The effect of mixture of vit. A acid, L-ascorbic acid and vit. E succinate on the migration of nuclear DNA from rat liver treated with DAB is presented in Table 3. At all the chosen doses, there was a significant ($P<0.001$) decrease on the migration of DNA, indicating a significant advantage of multiple vitamin therapy (Table 3) over the individual vitamin therapy.

### Discussion

Vitamin A deficiency is associated with the formation of various types of tumours. Retinol and retinoic acid have a number of biological functions including cellular differentiation and maintenance of normal integrity of mucosal tissues. In addition,
retinol prevents carcinogenesis through various mechanisms including inhibition of microsomal activation of chemical carcinogens. Consumption of alcohol is commonly associated with deleterious effects, some of which are due to vit. A deficiency which aggravates alcohol-induced hepatic damage. Fetal nutrient deficiency of vit. A results not only from a poor dietary intake, but may also be aggravated due to the breakdown of retinol in the liver due to direct effect of ethanol. Thus, a supplementation in heavy drinkers has a role in preventing liver problems. Consumption of fruits and vegetables rich in specific carotenoids and vitamins reduce the risk of pre-menopausal breast cancer. In the present study, all-trans-retinoic acids could protect DNA damage, whereas vit. A acetate and palmitate could not ward-off DAB-induced DNA damage in liver.

Free radicals exert their primary effects by reacting with macromolecules including DNA, lipids, proteins and carbohydrates. The oxidative DNA damage plays an important role in the carcinogenic processes induced by DAB, in addition to DNA adduct formation. Oxidation caused by free radicals is one of the major causes of DNA damage in humans. Abstraction of hydrogen atoms from unsaturated bonds in lipids is a major reaction of free radicals that results in lipid peroxidation ultimately giving rise to toxic products such as 4-hydroxyalkenals and malondialdehyde, which are considered to be a cause for carcinogenesis. The electrophilic nature of the metabolites of p-DAB has the capacity to covalently bind with RNA, DNA and tissue proteins. Vitamin C readily scavenges reactive oxygen and nitrogen species and may thereby prevent oxidative damage to important biological macromolecules such as DNA, lipid and proteins.

L-Ascorbic acid possesses substantial nucleophilic property and hence intercepts reactive agents or ascorbyl anion radicals, thus prevents their attack on nucleophilic sites and blocks the DNA damage. Its protective ability against DAB-induced hepatocarcinogenesis suggests it has anticancerous effect. The protective role of ascorbic acid against tumour may be due to its free radical scavenging, antioxidant, apoptosis-inducing and nucleophilic properties. The scavenging capacity of antioxidant vitamins is believed to prevent oxidative damage by neutralizing the free radicals.

In vitro experiments with DNA, nuclei and cells have shown that vit. C prevents DNA oxidation. Sodium ascorbate accelerates DNA damage in human cells in vitro. However, DNA damage in lymphocytes (measured by the comet assay and chromosome aberration test) is not affected after vit. C supplementation. Vit. C can protect human lymphocytes against DNA damage induced by oxidative agents such as hydrogen peroxide. It also protects DNA in human lymphocytes against damage induced by alloxan, paraoxon-methyl and parathion-methyl. Also, dose of as little as 10 mg of vit. C prevents scurvy. Recently, ascorbic acid has been found to have an antitumour effect in pancreatic cancer, when administered with a combination of lysine, proline, and green tea extract. Vit. C (sodium ascorbate) at 10 and 50 µM diminishes the DNA damage induced by selenium-cisplatin conjugate, but has no effect on the kinetics of DNA repair. In the present study, not all forms of vit. C, except L-ascorbic acid has shown the protective effect against DAB-induced DNA damage in liver.

The antioxidant supplementation of vit. C, vit. E, taurine, lutein, lycopene and β-carotene exerts a protective effect by decreasing the DNA damage. Preincubation of cells with vit. C or E at 10 or 50 µM causes a decrease in the level of DNA damage. Ascorbic acid prevents cytochrome P450-mediated oxidative damage of the nuclear membrane, but vit. E has shown no such effect. Liposomal formulations of vit. E acetate, given orally or by aerosol significantly reduces subcutaneous 66cl-4-GFP (BALB/c mammary tumour cell line) tumour burden and metastasis to lung and lymph nodes. Similar effect
on tumour burden and lung metastasis, but not on lymph node metastasis has been shown by the liposomal formulations of vit. E succinate delivered by aerosol.

In the present study, vit. E succinate delivered by gavage route has significantly reduced DNA damage, but vit. E and its acetate have failed to protect DAB-induced DNA damage in liver. Vit. E acetate is a potent inducer of apoptosis in a wide variety of human cancer cells, but not in normal cells in culture. We have also found that multiple vitamin therapy with vit. A acid, L-ascorbic acid and vit. E succinate is more effective in reducing DAB-induced DNA damage at low doses than treatment with individual vitamins of A, C or E, which may be due to their synergistic effect.

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