Modulatory effects of different doses of alpha-tocopherol on benzo(a)pyrene-DNA adduct formation in the pulmonary tissue of cigarette smoke inhaling mice

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Cigarette smoke (CS) has been established as one of the major risk factors for many pathologies including lung cancer in humans and experimental animals. In view of the discrepancy about the role of alpha-tocopherol (AT) in carcinogenesis, the present study was designed to investigate the effects of different doses of AT on benzo(a)pyrene-DNA [(B(a)P-DNA)] adduct formation in lungs of CS inhaling mice. Extent of carcinogen-DNA adduct formation has been considered as an index for carcinogenesis. Feeding of 35 IU AT/kg body weight increased B(a)P-DNA adducts formation significantly whereas feeding of 5 IU AT/kg body weight did not altered much the B(a)P-DNA adduct levels when both were compared to the control counterparts. With CS inhalation, the B(a)P-DNA adducts formation increased in all the groups when compared to their respective sham counterparts. Interestingly, in CS exposed groups, there was least increase in B(a)P-DNA adducts formation in 5 IU AT/kg fed animals followed by the control and 35 IU AT/kg body weight fed groups respectively. The results suggest that higher doses of AT accentuate DNA adduct formation in CS inhaling mice.

Keywords: Alpha-tocopherol; Benzo(a)pyrene; Cigarette smoke; DNA adducts

Epidemiological studies have established a causal relationship between cigarette smoking and various sites of human cancers, especially lung cancer. Cigarette smoke (CS) contains over 4700 chemical compounds including polycyclic aromatic hydrocarbons (PAHs) which are potent carcinogens and also a large amount of oxygen radical forming substances, such as catechol and hydroquinone which may enhance lung carcinogenesis by free radical-mediated reactions.

The PAH, benzo(a)pyrene (B(a)P), a well-known carcinogen, has been reported to induce tumors of various organs in experimental animals and is also linked to the etiology of human cancers through its presence in the environmental mixtures, vehicle exhausts and CS. It is a pro-carcinogen and requires metabolic activation to form reactive intermediates (the ultimate carcinogens) through the action of microsomal mixed function oxidases (MFOs) enzymes for eliciting its carcinogenic action. Following metabolic activation, it is converted into (±)-anti-7, 8-dihydroxy-9, 10-oxy-7, 8, 9,10-tetrahydroxy benzo(a)pyrene intermediate that covalently binds with DNA resulting in the formation of DNA adducts. These adducts may result in gene mutations upon replication of DNA and certain mutations particularly those resulting in activation of proto-oncogenes or inactivation of tumor suppressor genes are thought to play a critical role in the initiation phase of carcinogenesis. As it has been reported that the degree of carcinogenicity is directly proportional to the extent of DNA adduct formation, the extent of DNA adduct formation can be taken as an index for initiation of carcinogenesis.

While CS is of key importance, factors like diet also play a role in the development of lung cancer. Diet has been well-known to be able to raise or lower the risk of lung cancer. Dietary vitamins with antioxidant activity have received much attention in this regard. Among these alpha-tocopherol (AT) is of much importance, as it exhibits both antioxidant and pro-oxidant activities, but its role in carcinogenesis is still not well understood.

There are reports, which suggest that AT shows protective effects against the development of lung cancer. Also, Ichikawa et al. and Yano et al. have shown that that AT can act as a useful agent to protect mice from oxy-radical promoted lung tumorigenesis. However, Caraballosa et al. in a recent review has stated that antioxidant vitamins...
such as AT, beta-carotene or retinol, alone or in combination have no role in preventing lung cancer rather two of these, beta-carotene and retinol, at pharmacological doses causes harmful effects in smokers. Moreover, at higher doses AT act as a complete tumor promoter through free radical mediated mechanisms. The above observations prompted us to study whether, AT, at different doses, could have effect on the initiation phase of carcinogenesis too.

Keeping the above information in view, the present study has been designed to explore the effect of different doses of AT on the B(a)P-DNA adduct formation in the pulmonary tissues of cigarette smoke inhaling mice.

Materials and Methods

Animal model and treatments—Male Balb/c mice, weighing 20-30 g each, were procured from the Central Animal House, Panjab University Chandigarh. Animals were housed in clean polypropylene cages bedded with rice husk and were given free access to standard animal pellet diet (which contained all the essential nutrients including 100 IU/AT/kg of diet) and tap water throughout the experiment. After one week of acclimatization to the experimental conditions, the animals were divided into 6 groups of 8-10 animals each and were given various treatments as shown below:

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
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<tbody>
<tr>
<td>I</td>
<td>0.1 ml mustard oil (n=8)</td>
</tr>
<tr>
<td>II</td>
<td>0.1 ml mustard oil + CS exposure (n=10)</td>
</tr>
<tr>
<td>III</td>
<td>5 IU AT/kg body weight in 0.1 ml mustard oil (n=8)</td>
</tr>
<tr>
<td>IV</td>
<td>5 IU AT/kg body weight in 0.1 ml mustard oil + CS exposure (n=10)</td>
</tr>
<tr>
<td>V</td>
<td>35 IUAT/kg body weight in 0.1 ml mustard oil + CS exposure (n=8)</td>
</tr>
<tr>
<td>VI</td>
<td>35 IUAT/kg body weight in 0.1 ml mustard oil + CS exposure (n=10)</td>
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AT and/or mustard oil (vehicle) treatments were given orally daily with the help of a blunt tipped canula.

Mice of the smoke groups were subjected to passive cigarette smoke inhalation from commercial filter tipped cigarettes one hour daily in an inhalation apparatus designed in our laboratory and described earlier. This procedure has been standardized in such a way that animals inhale cigarette smoke without any visible respiratory stress as is evident from carboxy-haemoglobin (COHb) levels. It was observed that the CO levels in the control and CS-exposed animals were 1.2 ± 0.8 and 12.2 ± 4.9 percent saturation of haemoglobin, respectively. No doubt due to cigarette smoke inhalation there was an increase in the COHb levels in the smoke exposed animals as compared to their sham counterparts. However, the values found were within range that have been normally seen in CS smoke exposed animals. Clean and hygienic conditions were maintained throughout the experiment. Body weights of animals, feed and water intake by them were recorded daily.

B(a)P-DNA adduct analysis—After completion of the respective treatments, the mice were injected (i.p.) with 140 µCi of 3H-B(a)P /100 g body weight (specific activity 8000 mCi/mMole). After 12 hr of 3H-B(a)P injection, the animals were sacrificed by exsanguinations under light ether anaesthesia. After dissecting the thoracic cavity, lungs were perfused with ice-cold normal saline, weighed and 6% homogenate was prepared in 0.15 M NaCl solution containing 10 mM EDTA and 1% sodium dodecyl sulphate (SDS), pH 8.0. Pulmonary DNA was extracted by the method as described by Marmur. In short, pulmonary homogenates were emulsified with 1 volume of buffer saturated phenol. The emulsion thus prepared was centrifuged at 10,000 g for 20 min. Nucleic acids present in the aqueous phase were precipitated with 3 volumes of chilled ethanol. This solution was de-proteinised with Savag mixture (24 parts of buffer saturated chloroform + one part of isoamyl alcohol) and buffer saturated phenol in 1:1 ratio. After centrifugation at 10,000 g for 10 min and aqueous layer was taken. This layer was again de-proteinised by Savag mixture and centrifuged at 10,000 g for 10 min. The aqueous layer was taken and added 0.1 volumes of 5M NaCl. Finally the nucleic acid solution was extracted with 1 volume of chilled ethanol. After evaporating the residual ethanol, the nucleic acid residues were treated with 50 µg RNAase/ml (pre-incubated at 80°C for 10 min) and incubated at 37°C for 30 min in order to remove RNA. Nucleic acids were further extracted with 2 ml of Savag mixture. Finally DNA was precipitated out from the aqueous phase with chilled ethanol and redissolved in buffer. DNA contents were measured at 260 nm and purity of DNA extracts were checked by monitoring A260/A280 ratio. A ratio of A260/A280 >1.9 was taken as an index for DNA purity to be 99%. For determination of 3H-B(a)P bound to DNA, 0.6 ml of the DNA aliquots were transferred to toluene based
scintillation fluid containing 2,5-diphenyl oxazole (PPO) and 1,4-bis-2-(5-phenyloxazole)-2,5-phenyl-oxazole (POPOP). $^3$H activity was measured on a β-scintillation counter. Standards of $^3$H-B(a)P were also counted for calculation purposes.

**Statistical analysis**—The results were expressed as the mean ± SD of 8-10 animals in each group. For statistical significance, the data was analyzed by ANOVA followed by Student's Newman Keul test.

**Results and Discussion**

A non-significant change in B(a)P-DNA adduct formation in the pulmonary tissue was observed upon administration of 5 IU AT/kg body weight as seen in group III when compared with its control counterpart i.e. group I. However, a significant increase (1.6-folds) in B(a)P-DNA adduct formation occurred on administration of 35 IU AT/kg body weight as was observed in group V when it was compared to group I. Even a significant increase (1.3-folds) in B(a)P-DNA adduct formation was observed in group V when compared to the group III (Table 1).

CS exposure caused a significant increase in the B(a)P-DNA adduct formation in the pulmonary tissue (1.9-folds) as observed in group II when it was compared to its sham counterpart. A 1.3-folds increase in B(a)P-DNA adduct formation was also observed in the mice exposed to CS inhalation and fed with 5 IU AT/kg body weight (group IV animals) when compared to group III. However, this increase in B(a)P-DNA adduct formation was not much pronounced as observed in group II when compared to its sham counterpart. Interestingly, when the animals receiving a dose of 35 IU AT/kg body weight were given simultaneous exposure of CS, a 2.6-fold increase in B(a)P-DNA adduct formation occurred as observed in group VI when compared to the group V animals (Table 1).

Moreover, in CS exposed groups, it is very clear that B(a)P-DNA adduct formation is maximum in group VI whereas least B(a)P-DNA adduct formation has been observed in group IV. This indicates that at higher doses such as 35 IU/kg b.wt, AT is enhancing the B(a)P-DNA adduct formation in pulmonary tissues while at comparatively lower doses such as 5 IU/kg body weight, AT is inhibiting the same (Table 1).

It is well-known that B(a)P, a potent PAH present in CS, is a pro-carcinogen and requires metabolic activation mediated by mixed function oxidase (MFOs) enzymes for the generation of several of its active metabolites or ultimate carcinogens among which (±)-anti-7, 8-dihydroxy-9, 10-oxo-7, 8, 9, 10-tetrahydroxy B(a)P is the most active one, which binds covalently with cellular macromolecules 27. Although this active metabolite binds with proteins and RNA also, its interaction with nucleophilic sites in DNA to form adducts has been established as a critical event as far as the initiation of carcinogenesis is concerned 28. These adducts, if not repaired may leads to gene mutations upon replication of DNA and certain mutations particularly those resulting in activation of proto-oncogenes or inactivation of tumor suppressor genes are thought to play a critical role in the initiation phase of carcinogenesis 3. As the degree of carcinogenicity is directly proportional to the extent of DNA adduct formation 27, in the present study the extent of DNA adduct formation has been taken as an index of carcinogenesis.

Effect of different doses of AT and/or CS inhalation for eight weeks on *in vivo* B(a)P-DNA adduct formation, is shown in the Fig. 1.

In the present study, feeding of 5 IU AT/kg body weight did not caused significant alteration in B(a)P-DNA adduct levels in pulmonary tissue, whereas, at higher doses such as 35 IU AT/kg body weight, increased levels of the same were observed. This observation suggested that at higher doses, AT enhances B(a)P-DNA adduct formation. These changes may be due to the alterations in the activities of MFOs by excessive feeding of AT, as the activity of MFOs increase with AT administration in a dose dependent manner while the glutathione-S-transferase activity remains unaltered 31. With the increase in activity of MFOs, there could be an increase in the generation of active metabolites of the pro-carcinogens and as the activity of GST remains unaltered.

### Table 1—Effect of AT and/or CS on $^3$H-B(a)P-DNA adducts formation in lungs

<table>
<thead>
<tr>
<th>Group</th>
<th>$^3$H-B(a)P DNA adduct levels (fmols/μg DNA)</th>
<th>Statistical significance (q-values)</th>
</tr>
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<tbody>
<tr>
<td>I</td>
<td>31.7±9.0</td>
<td>-</td>
</tr>
<tr>
<td>II</td>
<td>61.0±11.2**</td>
<td>9.911 (II vs I)</td>
</tr>
<tr>
<td>III</td>
<td>38.2±2.6</td>
<td>2.086 (III vs I)</td>
</tr>
<tr>
<td>IV</td>
<td>48.8±9.4*</td>
<td>3.585 (IV vs III); 5.377 (IV vs II)</td>
</tr>
<tr>
<td>V</td>
<td>51.4±11.8*</td>
<td>6.321 (V vs I)</td>
</tr>
<tr>
<td>VI</td>
<td>133.2±4.9**</td>
<td>27.668 (VI vs V); 25.903 (VI vs II)</td>
</tr>
</tbody>
</table>

*, **: Same as in the Fig. 1
comparatively higher doses and simultaneous exposure to cigarette smoke is sure to contain numerous cytochrome P-450 inducers, a well-known component of MFOs. Also a highly significant increase in B(a)P-DNA adduct formation (2.6-folds) occurred when the animals receiving a dose of 35 IU AT/kg body weight were simultaneously exposed to CS, as observed in group VI when compared to the sham group i.e. group I. These results are supported by the earlier reports which showed increased formation of B(a)P-DNA adducts on exposure to CS\textsuperscript{31,32}. This may also be due to the increased activities of MFOs as it has been reported that CS exposure results in increase in the activities of MFOs\textsuperscript{33,34}. CS has been reported to contain numerous cytochrome P-450 inducers\textsuperscript{35}, a well-known component of MFOs. Also a highly significant increase in B(a)P-DNA adduct formation (2.6-folds) occurred when the animals receiving a dose of 35 IU AT/kg body weight were simultaneously exposed to CS, as observed in group VI when compared to the sham group. But interestingly a comparatively less significant increase in B(a)P-DNA adduct formation (1.3 times) was also observed in the animals which were maintained on a dose of 5 IU AT/kg body weight and simultaneously exposed to CS as observed in group IV animals when compared to the animals of group III.

It has also been reported that AT feeding in comparatively higher doses and simultaneous exposure to CS inhalation increases the activity of aryl hydrocarbon hydroxylase (AHH), one of the MFOs, in the pulmonary tissues while leaving the activities of other biotransformation enzymes unaffected\textsuperscript{36}. As explained earlier, the increased activities of AHH may be responsible for the increase in B(a)P-DNA adduct formation as observed in excessive AT fed animals simultaneously receiving CS exposure.

Moreover, among the CS exposed groups, maximum B(a)P-DNA binding has been observed in the animals receiving 35 IU AT/kg body weight i.e. group VI, while the B(a)P-DNA binding was found to be least in the animals which were fed 5 IU AT/kg body weight i.e. group IV. These observations suggest that CS inhalation accentuates B(a)P-DNA adduct formation, however, when animals were fed on 5 IU AT/kg body weight and were simultaneously exposed to CS inhalation, the B(a)P-DNA adduct formation was least, when animals of all the CS exposed groups were compared.

The present observations regarding the least increase in B(a)P-DNA adduct formation in CS inhaling, 5 IU AT/kg body weight fed animals could be due to the direct interaction of AT with the ultimate carcinogens at some optimum doses (5 IU AT/kg body weight) as is reported in case of butylated-hydroxy-anisole (BHA) where it has been shown to interact directly with ultimate carcinogens\textsuperscript{37} and resulting in a decreased B(a)P-DNA adduct formation. Also, possibly feeding of 5 IU AT/kg body weight may be modulating the MFO such that least reactive carcinogenic metabolite is available for the pulmonary DNA to bind. However, extensive work in this direction is in progress in our laboratory.

In conclusion, the present results indicate that excessive AT administration enhances B(a)P-DNA adduct formation. Although CS exposure also enhances the B(a)P-DNA adduct formation in all the groups studied, the enhancement of B(a)P-DNA adduct formation in the 5 IU AT/kg body weight fed CS exposed group was least as compared to control CS exposed group and 35 IU AT fed CS exposed group. These observations seem to be important as far as the initiation of carcinogenesis is concerned.

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