

Research article

## Effect of alpha-tocopherol on pulmonary antioxidant defence system and lipid peroxidation in cigarette smoke inhaling mice

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### Abstract

**Background:** Free radicals generated in biological systems by cigarette smoke (CS) inhalation can cause oxidative stress in tissues, resulting in lipid peroxidation (LPO). In view of the antioxidant properties of  $\alpha$ -tocopherol (AT), in the present study, effects of AT on antioxidant defence system and LPO were investigated in mice inhaling CS for different time intervals.

**Results:** Male Balb/c mice were fed orally with AT (5 I.U./Kg.b.wt.) and /or exposed to CS for 2, 4, 6 or 8 weeks. No effect was observed on body growth, diet consumption, water intake and lung weight due to AT and /or CS treatment in any of the groups as compared to their control counterparts. After two weeks of treatment, no change in LPO, reduced glutathione (GSH) levels and antioxidant enzymes were observed except for glutathione reductase (GR) which increased in all the treated groups. A significant increase in pulmonary LPO levels was observed in mice exposed to CS inhalation for 4, 6 or 8 weeks. There was a gradual increase in the LPO levels as the extent of CS inhalation increased from 4 to 8 weeks. However, the extent of increase in LPO levels due to CS exposure for 4, 6 or 8 weeks in the mice treated with AT was comparatively less. A significant decrease in the GSH levels was also observed in all the animals exposed to CS for 4, 6 or 8 weeks. There was a significant increase in the activities of catalase, glutathione peroxidase (GSH-Px) and GR observed in all the groups exposed to CS for 4,6 or 8 weeks. The increase in above antioxidant enzymes seems to be insufficient to combat the oxidative stress posed by CS inhalation. There was a marked decrease observed in the LPO levels in the animals treated with AT alone for 4, 6, or 8 weeks, when compared to their control counterparts. However, the supplementation of AT for 4, 6 or 8 weeks demonstrated a significant increase in GSH levels.

**Conclusion:** It appears from our studies that AT exhibits its antioxidant role either directly by scavenging the oxidative species or indirectly by modulating the GSH levels.

### Background

Cigarette smoke is a heterogeneous aerosol, which contains more than 4000 chemicals [1]. These include various compounds, which are capable of causing an increase in the generation of various reactive oxygen spe-

cies like  $O_2^{\cdot-}$ ,  $H_2O_2$ ,  $OH^{\cdot}$ ,  $ROO^{\cdot}$ . These reactive oxygen species in turn are capable of initiating and promoting oxidative damage in the form of lipid peroxidation [2,3]. Cigarette smoking may thus be associated with an increase in the incidence and severity of various diseases

like cancer, chronic obstructive lung disease and atherosclerosis [4].

Various organs may control or prevent the damaging effects of the oxidant species by enzymatic and non-enzymatic antioxidant defence systems. These include enzymes like superoxide dismutase (SOD), catalase and GSH-Px. Certain vitamins like  $\alpha$ -tocopherol [5] and ascorbic acid [6] are also suggested to have a strong free radical scavenging properties. The deleterious effects of the free radicals are kept under check by a delicate balance between the rate of their production and elimination by the different antioxidant systems. Any shift in this critical balance could result in an increase in the oxidative stress and may lead to cellular damage.

Although there is not enough evidence that indicates increased intake of antioxidant nutrients is beneficial, the observation that smokers have lower circulating levels of certain nutrients raises concern [4]. There is evidence, which suggests that supplementation with antioxidants like AT, might protect smokers from oxidative damage and could reduce risk from cancer or other diseases

caused by free radicals associated with smoking [7]. However, there are reports suggesting that the supplementation of vitamins like AT might not have a beneficial role in combating oxidative stress [8]. Therefore, the present study was designed to investigate the antioxidant role of AT in lungs of mice exposed to CS insult for varying duration.

## Results

There was no significant effect observed on body growth, organ weight, daily dietary consumption and water intake with CS inhalation and/or AT supplementation for 2, 4, 6 or 8 weeks in balb/c mice. Two weeks of CS inhalation and/or AT treatment did not show any significant difference in LPO, GSH levels and activities of catalase, GSH-Px or SOD when inter group comparisons were made. However, CS inhalation showed a significant increase in the GR activity in both AT + smoked and only smoked group. Moreover, a significant increase in GR activity was also observed with AT supplementation (from  $7.40 \pm 0.40$  to  $8.51 \pm 0.33$  nmole NADPH consumed  $\text{min}^{-1}$  mg protein $^{-1}$ ) (Table 1).

**Table 1: Effect of cigarette smoke and/ or AT on pulmonary antioxidant defence system in male balb/c mice over 2 weeks of treatment**

Parameters	Sham	Smoked	AT	Smoked + AT
LPO	16.05 $\pm$ 0.56	16.24 $\pm$ 0.49	15.72 $\pm$ 0.52	15.91 $\pm$ 0.68
GSH	3.04 $\pm$ 0.06	2.92 $\pm$ 0.06	3.03 $\pm$ 0.08	2.96 $\pm$ 0.03
Catalase	30.40 $\pm$ 0.99	33.19 $\pm$ 1.24	32.61 $\pm$ 1.12	35.24 $\pm$ 2.35
GSH-Px	10.02 $\pm$ 0.27	10.01 $\pm$ 0.32	9.42 $\pm$ 0.29	9.52 $\pm$ 0.29
GR	7.40 $\pm$ 0.40	8.56 $\pm$ 0.28 <sup>a</sup> <sub>1</sub>	8.51 $\pm$ 0.33 <sup>c</sup> <sub>1</sub>	9.26 $\pm$ 0.22 <sup>b</sup> <sub>1</sub> <sup>d</sup> <sub>1</sub>
SOD	2.90 $\pm$ 0.12	2.92 $\pm$ 0.66	2.60 $\pm$ 0.38	2.79 $\pm$ 0.53

Values are in mean  $\pm$  SD, n = 6–8 \* Units are expressed as follows : Catalase (I.U. mg protein $^{-1}$ ); GSH-Px and GR (nmol NADPH consumed  $\text{min}^{-1}$  mg protein $^{-1}$ ); GSH ( $\mu\text{mol g tissue}^{-1}$ ); SOD (I.U. mg protein $^{-1}$ ); LPO (nmol malondialdehyde mg protein $^{-1}$  30  $\text{min}^{-1}$ ). Statistical analysis<sup>a</sup>Sham vs. Smoked, <sup>b</sup>AT vs. AT + Smoked, <sup>c</sup>Sham vs. AT, <sup>d</sup>Smoked vs. AT + Smoked <sub>1</sub>, <sub>2</sub>, <sub>3</sub>, <sub>1</sub>, <sub>2</sub>, <sub>3</sub> : p < 0.05; <sub>2</sub>, <sub>2</sub>, <sub>2</sub>, <sub>2</sub> : p < 0.01; <sub>3</sub>, <sub>3</sub>, <sub>3</sub>, <sub>3</sub> : p < 0.001.

**Table 2: Effect of cigarette smoke and/ or AT on pulmonary antioxidant defence system in male balb/c mice over 4 weeks of treatment**

Parameters	Sham	Smoked	AT	Smoked + AT
LPO	16.53 $\pm$ 0.81	23.99 $\pm$ 0.45 <sup>a</sup> <sub>3</sub>	13.96 $\pm$ 0.70 <sup>c</sup> <sub>1</sub>	18.47 $\pm$ 0.56 <sup>b</sup> <sub>2</sub> <sup>d</sup> <sub>3</sub>
GSH	3.11 $\pm$ 0.02	2.80 $\pm$ 0.02 <sup>a</sup> <sub>3</sub>	3.27 $\pm$ 0.03 <sup>c</sup> <sub>1</sub>	2.68 $\pm$ 0.04 <sup>b</sup> <sub>3</sub> <sup>d</sup> <sub>2</sub>
Catalase	29.78 $\pm$ 1.65	39.01 $\pm$ 1.64 <sup>a</sup> <sub>1</sub>	34.51 $\pm$ 1.29 <sup>c</sup> <sub>1</sub>	39.18 $\pm$ 3.02 <sup>b</sup> <sub>1</sub>
GSH-Px	10.50 $\pm$ 0.27	15.05 $\pm$ 1.04 <sup>a</sup> <sub>2</sub>	7.46 $\pm$ 0.52 <sup>c</sup> <sub>2</sub>	11.13 $\pm$ 0.28 <sup>b</sup> <sub>3</sub> <sup>d</sup> <sub>2</sub>
GR	7.06 $\pm$ 0.11	10.96 $\pm$ 0.18 <sup>a</sup> <sub>3</sub>	9.82 $\pm$ 0.19 <sup>c</sup> <sub>3</sub>	12.36 $\pm$ 0.18 <sup>b</sup> <sub>3</sub> <sup>d</sup> <sub>2</sub>
SOD	2.84 $\pm$ 0.24	2.86 $\pm$ 0.24	2.95 $\pm$ 0.13	2.97 $\pm$ 0.14

Values are in mean  $\pm$  SD, n = 6–8 \*Same as in Table 1

Table 2 depicts the effects of CS inhalation and /or AT supplementation on pulmonary antioxidant enzymes after 4 weeks of treatment. In this phase of study it was observed that CS inhalation triggered LPO both in smoked and AT + smoked group. However, the extent of increase in LPO levels was comparatively less in the AT + smoked group. LPO levels also showed a significant decline from  $16.53 \pm 0.81$  to  $13.96 \pm 0.70$  nmoles MDA mg protein<sup>-1</sup> 30 min<sup>-1</sup> when mice were treated with AT (5 I.U./kg b.wt) for 4 weeks. Observations regarding GSH were exactly opposite to that of LPO in this phase of study. CS inhalation decreased the levels of GSH in both the smoked and AT + smoked group, whereas an increase in the levels of GSH was observed in AT supplemented animals as compared to their control counterparts. CS inhalation induced catalase, GSH-Px and GR activities without affecting the SOD activity in the smoked group as well as AT + smoked group. AT supplementation enhanced GR and catalase activities as compared to their controls, whereas a decline in GSH-Px from  $10.50 \pm 0.27$  to  $7.46 \pm 0.52$  nmole NADPH consumed min<sup>-1</sup>mg protein<sup>-1</sup> was observed in this group.

Table 3 depicts extension of CS inhalation and /or AT administration from 4 to 6 weeks. It showed similar effects

on LPO and GSH levels and other antioxidant enzymes studied as were seen after 4 weeks of treatment. 8 weeks of supplementation of AT and CS inhalation showed similar trends as seen after 4 and 6 weeks of treatment (Table 4). However, no increase in catalase activity was seen in the group treated with AT for 8 weeks. Moreover, CS inhalation also could not induce GR activity in the AT + smoked group as compared to AT group.

### Discussion

Cigarette smoke has been implicated as a major risk factor in many diseases such as pulmonary, cardiovascular pathologies. The adverse action of the CS is due to the presence of a large variety of compounds like nicotine, benzo (a) pyrene, oxidants, and free radicals that could initiate, promote and/or amplify oxidative damage. Although, CS is a major determinant of diseases related to oxidative stress, the inter individual variations in incidence and extent of these diseases are many [9]. Reasons like genetic susceptibility, extent of exposure to CS and/or presence of certain antioxidant micronutrients like AT in the diet of the individuals could be attributed to these variations.

**Table 3: Effect of cigarette smoke and/ or AT on pulmonary antioxidant defence system in male balb/c mice over 6 weeks of treatment**

Parameters	Sham	Smoked	AT	Smoked + AT
LPO	16.03 ± 0.38	25.60 ± 0.49 <sup>a</sup> <sub>3</sub>	14.37 ± 0.33 <sup>c</sup> <sub>2</sub>	19.01 ± 0.46 <sup>b</sup> <sub>3</sub> <sup>d</sup> <sub>2</sub>
GSH	3.06 ± 0.03	2.54 ± 0.03 <sup>a</sup> <sub>3</sub>	3.97 ± 0.04	2.65 ± 0.02 <sup>b</sup> <sub>2</sub>
Catalase	28.6 ± 0.95	38.65 ± 1.76 <sup>a</sup> <sub>2</sub>	36.02 ± 2.58 <sup>c</sup> <sub>1</sub>	41.33 ± 2.04 <sup>b</sup> <sub>1</sub>
GSH-Px	9.17 ± 0.13	16.73 ± 0.60 <sup>a</sup> <sub>3</sub>	6.52 ± 0.42 <sup>c</sup> <sub>2</sub>	11.34 ± 0.87 <sup>b</sup> <sub>2</sub> <sup>d</sup> <sub>2</sub>
GR	7.58 ± 0.26	12.75 ± 0.49 <sup>a</sup> <sub>3</sub>	11.21 ± 0.10 <sup>c</sup> <sub>3</sub>	12.19 ± 0.09 <sup>b</sup> <sub>3</sub>
SOD	2.71 ± 0.06	2.78 ± 0.13	2.77 ± 0.38	2.97 ± 0.46

Values are in mean ± SD, n = 6–8 \*Same as in Table 1

**Table 4: Effect of cigarette smoke and/ or AT on pulmonary antioxidant defence system in male balb/c mice over 8 weeks of treatment**

Parameters	Sham	Smoked	AT	Smoked + AT
LPO	16.07 ± 0.41	29.92 ± 0.45 <sup>a</sup> <sub>3</sub>	14.03 ± 0.35 <sup>c</sup> <sub>2</sub>	20.89 ± 0.56 <sup>b</sup> <sub>3</sub> <sup>d</sup> <sub>3</sub>
GSH	2.94 ± 0.05	2.23 ± 0.02 <sup>a</sup> <sub>3</sub>	3.76 ± 0.02 <sup>c</sup> <sub>1</sub>	2.37 ± 0.03 <sup>b</sup> <sub>3</sub> <sup>d</sup> <sub>2</sub>
Catalase	27.38 ± 0.97	37.71 ± 1.92 <sup>a</sup> <sub>2</sub>	28.29 ± 1.06	43.13 ± 6.22 <sup>b</sup> <sub>1</sub>
GSH-Px	9.79 ± 0.23	21.94 ± 0.33 <sup>a</sup> <sub>3</sub>	6.62 ± 0.44 <sup>c</sup> <sub>1</sub>	17.77 ± 0.74 <sup>b</sup> <sub>3</sub> <sup>d</sup> <sub>2</sub>
GR	6.88 ± 0.35	11.34 ± 0.63 <sup>a</sup> <sub>3</sub>	9.63 ± 0.09 <sup>c</sup> <sub>2</sub>	9.97 ± 0.16 <sup>d</sup> <sub>1</sub>
SOD	2.45 ± 0.08	2.62 ± 0.23	2.61 ± 0.60	3.01 ± 0.16

Values are in mean ± SD, n = 6–8 \*Same as in Table 1

In the first phase of investigation, where mice were subjected to CS inhalation and/or were fed with AT (5 I.U./kg.b.wt.) for 2 weeks, no significant change in pulmonary LPO, GSH content or any of the enzymatic antioxidants was observed, except for GR. An increase in GR activity was observed in all the groups studied. It seems that the treatment of CS inhalation for 2 weeks was not enough to pose any significant oxidative stress and thereby no increase in LPO. It may be possible that 2 weeks of CS was not enough to tilt the balance between the oxidative stress due to CS and the endogenous antioxidants. At the same time, AT supplementation for two weeks also seems to be unable to effect the levels of GSH. The changes in GR after 2 weeks of CS and AT seem to be a nonspecific response.

As the exposure of CS to the mice was extended from 2 to 4, 6 and 8 weeks, a significant increase in the LPO levels was observed. However, the extent of the increase in the pulmonary LPO levels in the AT + smoked group was comparatively low as compared to the animals not fed with AT and only exposed to CS. It was also observed that as the exposure to CS was extended from 4 to 6 and then to 8 weeks, there was a corresponding increase in the pulmonary LPO levels in both, the smoked as well as the AT + smoked group. However, the rate of increase of LPO in case of AT + smoked animals were comparatively less. These findings are suggestive of the fact that the increase in the duration of exposure to cigarette smoke, enhances the oxidative stress and thereby the LPO levels. However, AT appears to be responsible for the retarded increase of LPO in the AT + smoked group with increase in the duration of smoking.

In the sham exposed mice fed with AT for 4, 6 and 8 weeks, there was a significant decrease in the LPO levels observed as compared to their control counter parts. These observations too point towards the antioxidant properties of AT. Royack et al [10] have also reported that vitamin E as such may have the potential to reduce oxidative damage caused by hydroxyl free radicals.

In our studies, a significant decrease in the GSH levels was observed in both the smoked as well as AT + smoked groups and concomitantly there was an increase in the LPO levels. Therefore, the depletion of GSH seems to be associated with the increase of LPO. Younes and Siegers [11] have also observed that depletion of GSH enhances LPO. We have also observed that there was an increase in GSH levels in the AT fed group as compared to its control counterparts after 4, 6 and 8 weeks of treatment. On the other hand, in this group, LPO levels decreased indicating the antioxidant role of GSH in combating the oxidative stress. These findings correlate with our earlier report [12]. The unchanged levels of LPO in the presence

of unaltered GSH contents in the animals treated for 2 weeks with AT supports our view that levels of GSH play an important role in combating the oxidative stress.

We also observed that the other antioxidant enzymes like catalase, GSH-Px, and GR increased significantly in both the smoked as well as AT + smoked groups after 4, 6 and 8 weeks of CS exposure but still could not prevent the increase in LPO levels. It seems that this increase in the enzyme levels perhaps was a nonspecific response to the treatment or could be that this increase was not significant to combat the oxidative stress posed by CS exposure.

### Conclusions

It seems from our studies that AT plays an important role in combating the oxidative stress directly and/or through its affects on GSH levels. Moreover, studies related to the exact dose of AT, in order to quench oxidative stress in much more affective manner need to be worked on. Work in this direction is in progress in our laboratory.

### Materials and Methods

Male Balb/c mice weighing 20–25 gms, procured from the university animal house were used in the present study. Animals were fed on standard commercial pellet diet (Hindustan Lever Ltd) and had free access to water. After an acclimatization of one week, in our animal room, the animals were randomly divided into four groups and followed the treatment as shown in the Table 5.

**Table 5:**

S.No.	Group	Treatment
1.	Sham	0.1 ml of mustard oil/kg b.wt.
2.	Smoked	0.1 ml of mustard oil/kg b.wt + cigarette smoke inhalation.
3.	AT	5 I.U.α-tocopherol/ kg b.wt in 0.1 ml of mustard oil.
4.	AT+ smoked	5 I.U.α-tocopherol/kg b.wt in 0.1 ml of mustard oil + cigarette smoke inhalation.

*(Doses Standardized in our laboratory)*

AT or mustard oil (vehicle) were administered orally, everyday using a blunt tip canula. Mice in of the smoked groups were subjected to cigarette smoke inhalation as described earlier (13,14). In short 5–6 animals were exposed at a time to smoke from five commercially available filter tipped cigarettes. The procedure continued for 60 minutes every day in an inhalation chamber (8.21 lit-

ers). The chamber consisted of separate inlets for smoke and fresh air. After exposure of smoke from each cigarette, mice were exposed to only fresh air for a five minute interval and then another cigarette was lit. A valve attached at the outlet to a suction pump controlled the airflow (141 cc/min) through the chamber. Each cigarette took approximately 8 minutes to burn completely. No visible stress was observed on the animals while being exposed to the smoke. For comparison control animals (Sham exposed) were exposed to only fresh airflow in an identical chamber, where the cigarette was replaced by filter alone.

AT supplementation and cigarette smoke inhalation continued for either 2,4,6 or 8 weeks duration. After their respective treatments, mice from each group were sacrificed by exsanguination under light ether anesthesia between 7–8 hours after an over night fasting. The thoracic cavities of the animals were opened and the lungs excised and immediately perfused with cold 0.15 M KCl containing, 50 mM Tris-HCl buffer, pH 7.4. Tissues were homogenized and diluted to a concentration of 1 gm wet weight/4 ml of 0.05 M phosphate buffer, pH 7.4. Tissue homogenates were centrifuged at 10,000 × g for 20 minutes at 4°C. The supernatants thus obtained were used for different estimations. GSH-Px was assayed by the method described by Flohe and Gunzler [15]. Catalase activity was measured by the method of Luck [16]. SOD activity was determined by the method of Kono [17]. GR activity was assayed as reported by Carlberg and Mannervik [18]. GSH was estimated in the whole tissue homogenate by the method of Moron et al [19]. Lipid peroxide levels were assayed as described by Trush et al [20]. Protein was determined by the method of Lowry et al [21]. The statistical significance of inter group difference of various parameters were determined by unpaired students 't' test.

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