Original Research Article

Effect of tocopheryl acetate in neurobehavioral activity of cigarette of smoke exposed swiss albino mice

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ABSTRACT

Background: Cigarette smoke exposure is well known abuse which impairs neurobehavioral activity by oxidative damage to sensory and motor areas of cerebral cortex. Aim of the present study is to report the effect of tocopheryl acetate on oral administration in impaired neurobehavioral activity of cigarette smoke exposed Swiss albino mice.

Methods: Total thirty six adult Swiss albino mice were assigned into six different groups. Group I (n=6, distilled water and standard diet), group II (n=6, tocopheryl acetate induced), group III (n=6, soyabean oil induced as vehicle), group IV (n=6, cigarette smoke exposed), group V (n=6, cigarette smoke expose plus tocopheryl acetate), group VI (n=6, cigarette smoke plus soyabean oil). Frequency of cigarette smoke exposure was 3 times a day for 20 minutes each time and tocopheryl acetate with dose of 200mg/kg/day in 0.3ml of soyabean oil as vehicle orally through oral gavage for 28 days. On 29th day morning, the mice were subjected to perform neurobehavioral test such as open field tests and force swim test. After completion of the test, mice were sacrificed by cervical dislocation and brain was autopsied to estimate malondialdehyde (MDA), superoxide dismutase (SOD) and reduced glutathione (GSR) for oxidative level and histopathological examination of brain.

Results: The treated mice exposed to cigarette smoke showed decreased motor activity in open field test and increased anxiety in force swim tests. On histopathological examination, marked neuronal damage was observed in motor area of cerebral cortex. Oxidative level in neuronal tissue was highly variable by an increased level of MDA (815.2±56.62, p<0.0001) and decreased level of SOD (1.5±0.54, p<0.001) and GSR (0.025±0.007, p<0.001) as compared to control group. Administration of tocopheryl acetate improved the neurobehavioral activity and maintained oxidative level significantly (p <0.0001 in MDA, p <0.001 in SOD and GSR).

Conclusions: Tocopheryl acetate can prevent neuronal damage due to cigarette smoke exposure. Thus, it can be used as a protective agent for neurobehavioral impairment, neuronal cell damage and altered oxidative level occurring in cigarette smokers.

Keywords: Anxiety, Depression, Oxidants/Antioxidant, Neuroprotection, Neuropathogenesis

INTRODUCTION

Cigarette smoke consists of psychoactive complex mixture, being abused by one third populations of the world. It has been calculated that cigarette smoke contains 10^17 oxidant molecules per puff, of which 10^14 are reactive oxygen species such as superoxide radical and nitrogen oxide, both of which immediately react to form highly reactive peroxynitrite. Hydroquinones that undergo redox-cycle to form superoxide radical and hydrogen peroxide via semiquinones, thereby result in persistent oxidative stress. It is well known fact from various geographical surveys that cigarette smoking is a
major cause of neurobehaviour disorders.5-8 Impact of nicotine from cigarette smoke on the central nervous system is neuroregulatory, affecting biochemical and physiological functions. Dose-dependent neurotransmitter and neuroendocrine effects occur as plasma nicotine levels rise due to exposure of cigarette smoke either actively or passively.5

Circulating levels of norepinephrine and epinephrine increase, and the bioavailability of dopamine is altered as well.10 Among the neuroendocrine effects are release of arginine, vasopressin, beta-endorphin, adrenocorticotropic hormone, and cortisol. Notably, several of these neurochemicals are psychoactive and/or known to modulate behaviour such as anxiety and depression.11 On other hand, locomotor activity requires the coordinated actions of cortical and subcortical regions involving cerebrum cerebellum, brain stem and basal ganglia.12-14 Tocopheryl acetate, known as vitamin E acetate is an antioxidant. It is the ester of acetate and tocopherol, which on administration in vivo, fulfils the depleted level of antioxidants in cigarette smokers.15

The present study was conducted to identify how administration of tocopheryl acetate shows positive effects on neurobehavioral activity in swiss albino mice exposed to cigarette smoke, with due improvement in neuropathogenicity of brain and oxidative stress.

METHODS

The current Mice model experimental study was conducted in the Department of Anatomy, Institute of Medical Sciences, Banaras Hindu University, Varanasi-221005, India from March 2015 to April 2016. Adult Swiss albino mice with an average weight and age about 20-30gm and 60-90 days old respectively were used in the present study.

Procurement and acclimatization of animal

Swiss albino mice were procured from the animal house of Department of Anatomy, Institute of Medical Sciences, Banaras Hindu University. They were reared in polypropylene cage (25x 20x15cm) under standard laboratory condition (25°±5°C, 12hr L/D cycle, 55±5 Relative Humidity) with 2 weeks proper acclimatization. Dry rice bran was used as a bedding material.

They were fed pelleted diet obtained from animal feed supply center, Varanasi and tap water ad libitum. The experiment was done with utmost care to avoid pain and harm to mice such as room heater and towel were used during force swim test. The use of animals in this research was approved by and conducted in compliance with the guidelines of Animal Ethics Committee of Banaras Hindu University (reference Dean/2015/CABC/191) after submitting the synopsis where number of mice in each group was six.

Cigarette smoking system

To assume environmental tobacco smoke i.e. mainstream/second stream smoke for experimental mice, cigarette smoking system (CSS) protocol was modified from the exposure protocol of Santiago et al, in order to expose cigarette smoke in which CSS consisted of three chambers.11,16

Air generator: Air Generator was meant for generation of air inside the chamber and aeration to the smoke chamber through a 25cm long wind pipe. It had six walls with dimension of 15x10x10cm. Air cool fan with 6cm diameter consisting of 11 leaflets, 5V DC, 50mA adaptor motor was fixed on one of the walls. Opposite to this wall, a 25cm long wind pipe was fixed to connect the pipe to smoke chamber.

Smoke chamber

Smoke Chamber was meant for accumulation of cigarette smoke produced from cigarette sticks. This chamber consisted of six walls with dimension of 20x10x10cm. It consisted of an open/close able door (12x10cm) through which, cigarettes were taken in and out of the chamber. It had two holes; one hole on wall facing towards air generator to receive the wind pipe from air generator and another hole on its roof to fix a 60cm long wind pipe which connected to inhalation chamber. It also contained a cigarette stand which was used to stand burning cigarettes.

Inhalation chamber

Inhalation chamber was meant for cigarette exposure of mice with dimension of 50x25x20cm. It consisted of six walls on which an acrylic sheet was fixed on an open wall (40x13cm) to observe the behavioural activities of mice during their exposure. Roof was open/close able by two shutters to allow the smoke vapour out and to keep mice in/out of the chamber. A 60cm long wind pipe was passed through a wall facing towards the smoke chamber to receive cigarette smoke from smoke chamber.

Experimental design

Thirty six mice were taken in the present study and randomly assigned into 6 groups. Each group contained six mice as following; Group I (n=6, distilled water and standard diet), group II (n=6, tocopheryl acetate induced), group III (n=6, soyabean oil induced as vehicle), group IV (n=6, cigarette smoke expose), group V (n=6, cigarette smoke expose plus tocopheryl acetate), group VI (n=6, cigarette smoke plus soyabean oil). Groups IV, V and VI were exposed to cigarette smoke 3 times a day for 20 minutes each time which was optimum dose and duration of exposure for the most sustainable cigarette smoke environment in the study. Groups V and III were induced by tocopheryl acetate with dose of 200mg/kg/day
in 0.3ml of soyabean oil as vehicle orally through oral gavage for 28 days.

**Behavioural study**

Treated and control mice of each group were allowed to perform neurobehavioral activity on 29th day onwards by cognitive behavioral experiment such as open field test and force swim test in cognitive laboratory in Department of Anatomy at 9:00 AM.

**Open field test**

This open field test was done to test the activity, locomotor and anxiety related effects in all experimental mice. An open field apparatus made of plywood measuring 60.96 x 60.96 x 60.96cm was used to test open field exploratory behavior of mice. The floor of the apparatus was divided into 10 evenly spaced squares surrounded by opaque high walls of 60.96cm. The entire apparatus was painted black except for the 6mm wide white line that divided the floor into 16 squares. The open field was illuminated by 100W bulb focusing into field from height of about 100cm from the floor. The entire room except the apparatus was kept dark during experiment. Animals were kept for 5minutes to observe the frequency of line crossed, centre square entry, rearing, grooming, freezing and fecal boli.

**Force swim test**

This force swim test was done to observe anxiety or anti-depressant efficacy in all experimental mice groups. Forced swim test was done in cylindrical swim tank 45cm diameter and 25cm depth of tap water maintained at 25°C. The mice were brought into the cognitive laboratory at least 30minutes before beginning the test. Then the mice were gently placed in the middle of the tank and allowed to swim for 5minutes of session to observe immobility duration in second.

**Sacrifice of the mice and tissue collection**

After behavioral performance, treated and control mice of each group were sacrificed by cervical dislocation for histological observation and biochemical study. Immediately after cervical dislocation, brain was taken by removing maxilla, frontal, parietal bones and occipital bone with help of pointed forceps.

After dissection, unwanted residues of mice were disposed by burying in sterile soil. Thus, obtained brain was fixed in 10% formaldehyde solution at least for a week. Haematoxylin and eosin staining was done for histopathological observation in frontal cortex of the brain. Photo micrograph was taken by camera fitted Lynx, Lawrence and Mayo binocular microspore under 10X. The cell count was done by image J software (version 2016).

**Oxidative study**

Immediately after cervical dislocation, abdomen was exteriorized by mid line incision. Adequate quantity of brain tissues was autopsied, kept in phosphate buffer solution (PBS) at -20°C and 10% (w/v) tissues was homogenated in ice cold PBS (0.1M, pH 7.4). The homogenate was centrifuged, and the resulting supernatant was used for all the biochemical parameters to test the oxidative level.

**Estimation of malondialdehyde (MDA) level**

MDA was estimated by thiobarbituric acid test protocol from Devasagayam et al, at 530nm by ELICO- SL-104 double beam UL-UV Spectrophotometer.16

**Estimation of superoxide dismutase (SOD) level**

SOD was estimated on the inhibition of the formation of NADH-phenazine methosulphate-nitroblue tetrazolium formazan from modified protocol of Kakkar et al. The colour formed at the end of the reaction and measured at 560nm by ELICO-SL-104 double beam UL-UV Spectrophotometer.17

**Estimation of reduced glutathione (GSR) level**

GSR was estimated by development of yellow color when 5’s diethobis (2-nitrobenzoic acid) was added to sulphydryl compound. This reaction was read at 420nm by ELICO-SL-104 double beam UL-UV spectrophotometer.

**Statistical analysis**

All data were entered into excel sheet and mean and standard deviation (S.D.) were calculated. One way ANOVA followed by Bonferroni test was done using software Graph Pad Prism 5. Cell count was done by image J software for histocytometry. If it was significant, multiple comparison test was used for post hoc analysis in order to compare control and treated groups. P<0.05 was considered as significant and P<0.0001 was considered as highly significant.

**RESULTS**

**Open field test**

Locomotor activity, memory and anxiety was reported by open field test among controls and various treated mice groups. Score of line crossed, centre square entry, rearing and grooming had significantly decreased (p <0.0001) in cigarette smoke exposed mice group i.e. group IV in comparison to controls i.e. group I, II, and III. On administration of tocopherol acetate in cigarette smoke exposed mice i.e. group V there was significant increase (p <0.0001) in scores of lines crossed, centre square entry, rearing and grooming in comparison to group VI.
Freezing and fecal pellet had significantly increased (p < 0.0001) in group IV in comparison to groups I, II and III where group V had significantly decreased (p<0.0001) score of rearing in comparison to group IV (Table 1).

Table 1: Score obtained in open field test by different groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Line Crossed</th>
<th>Center square entry</th>
<th>Rearing</th>
<th>Grooming</th>
<th>Freezing</th>
<th>Fecal pellet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>63.00±4.82</td>
<td>2.17±0.41</td>
<td>12.50±1.76</td>
<td>3.00±0.89</td>
<td>5.83±1.47</td>
<td>1.50±1.05</td>
</tr>
<tr>
<td>Group II</td>
<td>64.50±9.42</td>
<td>1.50±0.84</td>
<td>12.83±1.84</td>
<td>3.66±1.21</td>
<td>5.00±1.09</td>
<td>1.17±0.75</td>
</tr>
<tr>
<td>Group III</td>
<td>60.00±9.34</td>
<td>1.50±0.55</td>
<td>12.17±1.72</td>
<td>3.00±0.89</td>
<td>5.17±0.98</td>
<td>2.00±0.63</td>
</tr>
<tr>
<td>Group VI</td>
<td>17.17±5.27***</td>
<td>1.00±0.63***</td>
<td>4.67±2.85***</td>
<td>6.16±0.75*</td>
<td>8.17±0.75**</td>
<td>4.50±1.04***</td>
</tr>
<tr>
<td>Group V</td>
<td>46.17±9.48</td>
<td>2.00±0.63</td>
<td>8.50±1.05</td>
<td>2.83±0.75</td>
<td>6.17±0.75</td>
<td>3.17±1.17</td>
</tr>
<tr>
<td>Group VI</td>
<td>16.33±5.75**</td>
<td>1.00±0.64**</td>
<td>3.83±1.47**</td>
<td>6.33±0.76**</td>
<td>7.83±1.17*</td>
<td>4.50±0.84</td>
</tr>
</tbody>
</table>

(*p<0.01, **p<0.001, ***p<0.0001)

Figure 1: Cerebral cortex of mice showing in control group I, II and III. Cigarette smoke exposed group IV showing perinuclear lacuna (black arrow), multiple nuclei cluster (yellow arrow), and fragmented nuclei in cortical cerebrum. Tocopheryl acetate administered in cigarettes smoke exposed group V showing few mitosed (yellow arrow) cell and abundantly perinuclear lacuna (black arrow). Cigarette smoke expose and soyabean oil orally administered group VI showing multiple nuclear cluster (yellow arrow), perinuclear lacuna (yellow arrow), mitosed and fragmented nucleus with shrunken cell.

Force swim test
In force swim test, immobility duration in second showed significantly to evaluate anxiety and antidepressant, after the oral administration of tocopheryl acetate in cigarette smoke exposed mice. Cigarette smoke exposed mice i.e. group IV had significantly increased (p <0.0001) immobility duration in comparison to control groups i.e. group I, II and III (Table 2). Immobility duration was significantly decreased (p <0.0001) in group V by
induction of tocopheryl acetate in cigarette smoke exposed group. Group I and II were non-significant in inter comparison as control (Table 2). Cigarette smoke exposed plus soyabean oil induced group (group VI) had significantly increased immobility (p<0.0001) in comparison to control but it was non-significant when compared to group IV (Table 2).

**Histopathological observation**

![Histopathological observation](image)

**Figure 2: Different experimental group cell counts by image J.**

On histopathological observation in motor area of cerebral cortex of frontal part of treated mice, it was reported that cigarette smoke exposed group i.e. Group IV and VI showed aggregated ependymal lining with multiple astrocyte clusters around the pericellular space in molecular layer.

In outer granular layer, multiple aggregated pyramidal cells were observed. Astrocyte which undergone mitosis in two celled stage were commonly seen in this layer. In outer pyramidal layer, multiple mitosis pyramidal cells with blabbing were commonly seen. In inner granular layer, multiple aggregated pyramidal cells with lacuna were seen (Figure 1).

On administration of tocopheryl acetate in cigarette smoke exposed i.e. group V no such findings were seen but few pericellular lacuna and mitosis undergone two celled stage cells were observed (Figure 2).

**Table 2: Immobility duration obtained in forced swim test by different groups.**

<table>
<thead>
<tr>
<th>Group</th>
<th>Immobility (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>131.00±11.33</td>
</tr>
<tr>
<td>Group II</td>
<td>153.50±9.18</td>
</tr>
<tr>
<td>Group III</td>
<td>142.70±12.42***</td>
</tr>
<tr>
<td>Group IV</td>
<td>230.30±15.03***</td>
</tr>
<tr>
<td>Group V</td>
<td>150.80±16.94***</td>
</tr>
<tr>
<td>Group VI</td>
<td>231.30±16.27***</td>
</tr>
</tbody>
</table>

***p<0.0001

**Table 3: MDA, SOD and GSR activity of different group of mice.**

<table>
<thead>
<tr>
<th>Group</th>
<th>MDA (µmol/mg)</th>
<th>SOD (µmol/mg)</th>
<th>GSR (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>278.5±32.98</td>
<td>3.17±0.41</td>
<td>0.038±0.005</td>
</tr>
<tr>
<td>Group II</td>
<td>279±52.05</td>
<td>3.33±0.81</td>
<td>0.035±0.006</td>
</tr>
<tr>
<td>Group III</td>
<td>276.00±31.14</td>
<td>3.83±0.98</td>
<td>0.033±0.005</td>
</tr>
<tr>
<td>Group IV</td>
<td>815.2±54.62***</td>
<td>1.5±0.54**</td>
<td>0.025±0.007**</td>
</tr>
<tr>
<td>Group V</td>
<td>570.5±56.32***</td>
<td>3±0.89</td>
<td>0.036±0.005</td>
</tr>
<tr>
<td>Group VI</td>
<td>823.5±68.17***</td>
<td>1.5±0.55**</td>
<td>0.021±0.006**</td>
</tr>
</tbody>
</table>

**Oxidative level in brain**

Oxidative level was assessed by the estimation of level of MDA, SOD and GSR from the brain tissue. Cigarette smoke exposed group i.e. group IV showed significantly increased level of MDA and decreased level of SOD and GSR in comparison to control group I, II and III (Table 3). On administration of tocopheryl acetate to cigarette smoke exposed mice i.e. group V, significantly decreased MDA level and increased SOD and GSR level was observed. MDA, SOD and GSR level in group I, II and III were not significant in inter comparison (Table 3) also Groups V and VI were not significant in inter comparison (Table 3).

**DISCUSSION**

Cigarette smoke is highly psychoactive component which can cause physiological behavioural changes on chronic cigarette smoke exposure (CSE).

In present study, CSE inhalation chamber was used which has capability of producing both mainstream and
side stream smoke for cigarette. Cigarette smoke exposure system delivered smoke to adult mice for 28 days (four weeks) which is comparable to human smoke exposure actively or passively. Therefore, Cigarette smoke exposed of mice is more relevant model.18

Present study also showed that locomotor activity such as line crossed and centre square entry in open field test were significantly decreased in cigarette smoke exposed mice group (CSE) IV and VI. On repeated tocopheryl acetate administration in group V the locomotor activity increased as compared to CSE group. Parameters observed for anxiety and depression such as rearing and grooming decreased significantly in group IV and VI whereas freezing and fecal pellet were significantly increased in CSE group IV and VI. In group V there was significant decrease in depression and anxiety parameters. Similar findings were reported by earlier researches in human and mice.19-21

Another behavioural test was performed by force swim test which represented anxiety and anti-depression efficacy by change in immobility duration. This was significantly increased in group IV and V, whereas group V showed significantly reduced immobility duration.

On microscopic examination, frontal cortex of cerebrum was found to undergo pathological changes such as perinuclear lacuna, multiple nuclei cluster and severe fragmentation in CSE group IV and VI. This observation showed the frontal cortex which is main area for controlling behavior was damaged due to CSE. Only few pathological cells were observed in group V. Such findings were not found in control group I, II and III.

On estimation of oxidative level, MDA was significantly increased whereas SOD, GSR were significantly decreased which showed markedly increased oxidative stress in CSE group IV and VI. On administration of non-enzymatic antioxidant tocopheryl acetate, significantly reduced MDA and increased SOD and GSR level were observed. Previous studies reported reduced levels of Dopamine, Norepineprine and 5-hydroxytryptamine in cerebrum, cerebellum and brainstem and other parts of brain such as substantia nigra, basal ganglia by disrupting nigrostrial pathway which consequence to impair in locomotort and anxiety.22 This reduction may be due to a central cholinergic excitation caused by accumulation of Acetylcholine due to drug-induced cholinesterase inhibition.23 This is in accordance with findings of Fiscus et al, in which another cholinesterase inhibitor parathion was shown to decrease norepinephrine and dopamine levels.24 Present study can be associated with cigarette smoke exposure and locomotor activity and anxiety. This can be due to poor establishment to and fro connection with thalamus, hypothalamus and many other regions of the cerebral cortex to integrate the function for behavioral control.25-26

The present study shows an association of the neuronal damage with oxidative level increased by cigarette smoke in mice. Decreased antioxidant level such as SOD and GSR in CSE mice were ameliorated by exogenous repeated dose determined tocopheryl acetate administration. Thus, tocopheryl acetate might play a role in improvement of impaired neurobehaviors such as anxiety and depression by correcting the oxidative level.

Limitation of the present work is the result of the study is based on animal model performances, these findings may not translate to case of human cigarette smoke exposure in determined duration in the study.

CONCLUSION

Repeated oral administration of Tocopheryl acetate in cigarette smoke exposed mice model improves the behavioural impairment by correcting the various oxidative pathways, which also repairs the neural damages in sensory-motor cortex of cerebrum that maintain the to and fro connection with other parts of the brain to control the anxiety, depression, memory and movement of the experimental mice. Therefore, tocopheryl acetate can be a good drug to maintain the altered level of oxidants and antioxidant and prevent neuronal damage of brain cause by cigarette smoke exposure.

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Ethical approval: The study was approved by the Institutional Ethics Committee

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