

# Protective role of vitamin E on the harmful effects of toluene on brain tissue

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## ABSTRACT

**Objective:** To determine the protective effects of vitamin E against the damage inflicted by reactive oxygen species during toluene induced brain injury in rats using histopathological, biochemical, and behavioral parameters.

**Methods:** Twenty-eight Wistar albino male rats were studied at Hacettepe University Faculty of Medicine Laboratories and Gazi University Faculty of Medicine Metabolism Laboratory in 2006. The rats were divided into 4 groups as follows: control group, toluene only treated group, both toluene and vitamin E treated group, and vitamin E only treated group. Histopathological changes were observed by hematoxylin and eosin staining and the TUNEL method. Serum lipid peroxide levels were measured by the thiobutyric acid method, and the open field test was used for behavioral testing.

**Results:** Our results show that edema between cells in the neuropil, particularly beneath the pia mater, and around congested blood vessels was observed in all groups with different severity. However the pyknotic neurons, in addition to infiltrative cells, were observed in the toluene group to be decreased by the administration of vitamin E, which can be confirmed by the euchromatic nucleated neurons in the cortex of both the toluene and vitamin E treated groups. The levels of malondialdehyde in each group also confirmed these histopathological findings. Significant differences were also found in the open field test between the groups.

**Conclusion:** The differences between the toluene and vitamin E groups in biochemical, histologic, and behavioral examinations, supports the antioxidant protective role of vitamin E against the harmful effect of toluene on the brain.

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Toluene is a well known neurotoxic and genotoxic chemical.<sup>1-3</sup> It has been shown to have neurobehavioral effects.<sup>4-6</sup> Inhalants are readily available in common household items, such as cleaning fluids, nail polish remover, paint thinner, gasoline, and adhesives. Toluene is a commonly abused drug. Users typically place the inhalant in a bag and inhale the fumes to elicit the desired effect.<sup>7</sup> The chronic abuse of toluene results in structural and functional impairment of a variety of organs such as the bronchial system and lungs.<sup>8</sup> Toluene fume inhalation is an important cause of encephalopathy, and may lead to irreversible brain damage. In particular, toluene abuse has been shown to cause permanent changes in brain structures that correlate with neural dysfunction.<sup>9,10</sup> It can cause CNS depression, loss of memory, and progressive brain and nerve damage.<sup>11</sup> Furthermore, Mattia et al<sup>12</sup> demonstrated that intraperitoneal injection of toluene caused a significant elevation in the rate of reactive oxygen species (ROS) generation, and a reduction in levels of reduced glutathione in the brain.<sup>12</sup> There are several studies reporting cellular damage of toluene via formation of ROS that are believed to cause lipid peroxidation and an increase in its product, malondialdehyde (MDA), resulting in damage to biological membranes.<sup>13-15</sup> Small molecule antioxidants, such as vitamin E, are able to interact with oxidizing radicals directly. Vitamin E terminates the chain reaction of lipid peroxidation in biomembranes and lipoproteins.<sup>16,17</sup> Lipid peroxidation is propagated to other sites causing widespread membrane damage and destruction of organelles. Degradation of membrane phospholipids progressively are the main constituent of the cellular injury. Disturbance in cell membrane function, because of alterations in the biochemical systems of cells, results in cellular injury. By the administration of vitamin E, lipid peroxidation can be inhibited, leading to the maintenance of

membrane stability and cellular constancy. This may be related to the fact that vitamin E, as a chain-breaking antioxidant, is thought to be a protective agent, mainly against membrane damage. Subsequently, we aimed to determine the protective effects of vitamin E on the harmful effects of toluene on rat brain tissue and behavior performance.

**Methods. Study design.** The subjects were 28 Wistar albino male rats (outbred stock from the Institute's breeding colony). The rats weighed 225-250 g (approximately 50-55 days old) at the beginning of the experiment, which was carried out at Hacettepe University, Faculty of Medicine Laboratories, and Gazi University, Faculty of Medicine Metabolism Laboratory in 2006. The rats were not subjected to any experimental procedure or exposed to any chemicals prior to the onset of this study. The rats were divided randomly into 4 groups: The control (C) group (n=7), toluene (TN) only group (n=7), the toluene + vitamin E (TN + Vit E) group (n=7), and the vitamin E (Vit E) only group (n=7). They were housed socially in standard type IV poly carbon cages (width x height x depth = 380 x 200 x 590 mm) with standard rat food pellets (Korkutelim Ltd), and water available ad libitum. The colony room was maintained at a temperature of 21±2°C and on a 12-hour:12-hour light/dark cycle (lights on at 7:00 a.m.). All experimental manipulations were conducted during the light phase. All animal-used protocols were in accordance with The European Communities Council Directive of November 1986 (86/609/EEC) and followed the regulations outlined by the Hacettepe University Committee on Laboratory Animals (2004/71-2). Blood samples were collected by cardiac puncture 24 hours after the end of toluene injection, and the rats were then sacrificed by decapitation.

**Chemicals.** The control group was treated for 5 days with daily intraperitoneal injections of 0.5 cc olive oil. Toluene was diluted with oil at a concentration of 1 ml/ml and administered intraperitoneally to the TN group (n= 7) at a dose of 1.3 ml/kg/day (Merck) for 5 consecutive days. The selected dose was half of the lethal dose, 50% (LD<sub>50</sub>) per day, 1.3 ml/kg toluene for the TN group, both 1.3 ml/kg and 10 mg/kg vitamin E (Evigen-AKSU) for the TN + Vit E group, and only 10 mg/kg vitamin E for the Vit E group.<sup>18</sup>

**Behavioral testing.** The behavioral testing began on the 5th day of the experiment. All behavioral testing occurred during the light phase of the light:dark cycle. The injections continued during the behavioral testing phase and were administered before the behavioral testing for each day was started (a minimum of one hour before the first rat was tested).

**Open-field test.** The open-field test is designed to measure behavioral responses such as locomotor activity,

hyperactivity, and exploratory behaviors. Open field is also used as a measure of anxiety. Rats tend to avoid brightly illuminated, novel, open spaces, so the open field environment acts as an anxiogenic stimulus and allows for measurement of anxiety-induced locomotor activity and exploratory behaviors. Open field testing is a one trial test with little or no impact on the animal's subsequent behavior. The apparatus for the open field test is a square floor (70 cm x 70 cm) and 60 cm high made of black wood with a transparent Plexiglas floor and no top.<sup>19,20</sup> In the present study, each rat was placed in a corner of the open-field and its behavior recorded for 5 minutes. The open-field was wiped with fresh water between the testing of each animal.<sup>19</sup> All activity was recorded by a video camera mounted above the open-field and scored in real-time. The video image of the open field arena is partitioned into 36 equal-size squares; 24 border squares and 12 center zone squares. We measured and analyzed total distance for measure of spontaneous locomotor activity levels, the number of line crosses into the center (squares not adjacent to the walls of the open field) percentage of time spent freezing in various parts of the open-field for anxiety. Anxious rats tend to make very few entries into the center of the open field.<sup>20</sup> Testing was carried out in a temperature, noise, and light controlled room.

**Histochemistry and immunohistochemistry for light microscopy.** Brain tissue specimens were immediately fixed in 10% buffered formaldehyde and processed according to routine histological techniques. Each specimen was prepared from the cerebral cortex within the same hemisphere. Ten sections of 5 µm stained with hematoxylin and eosin were examined for cortical architecture, ventricular integrity, presence of pyknotic cells, and glial cell count. Number of normal cells, pyknotic cells, and glial cells were counted by 2 blinded examiners in 10 consecutive high-power fields at 40X magnification by an oculometer, the average of their counts was recorded and cell numbers in a one mm<sup>2</sup> area were calculated. In the adjacent sections, DNA fragmentation was evaluated by the terminal transferase mediated d-UTP nick-end labelling (TUNEL) method (ApopTag Plus Peroxidase, In Situ Apoptosis Detection Kit; Q-Biogene, USA).<sup>21</sup> Methyl green was used for background staining. Two blinded examiners counted apoptotic cells in 10 high-power fields at 40X magnification by an oculometer, the average of their counts was recorded and cell numbers in a one mm<sup>2</sup> area were calculated. All sections were evaluated under Olympus BH2 light microscope (Tokyo, Japan).

**Biochemical assays.** Serum lipid peroxide levels were measured colorimetrically by the thiobutyric acid method, which was modified from the methods of Yagi as reported recently.<sup>22</sup> Malondialdehyde (MDA) results were expressed as nanomoles per milliliter.

**Statistical analysis.** The data were analyzed using SPSS version 11.5 for windows. For all analyses, the TN + Vit E rats were compared to the TN rats and control (C) rats. The differences among groups were estimated with the student's-t test. Probability values,  $p < 0.05$  were considered significant.

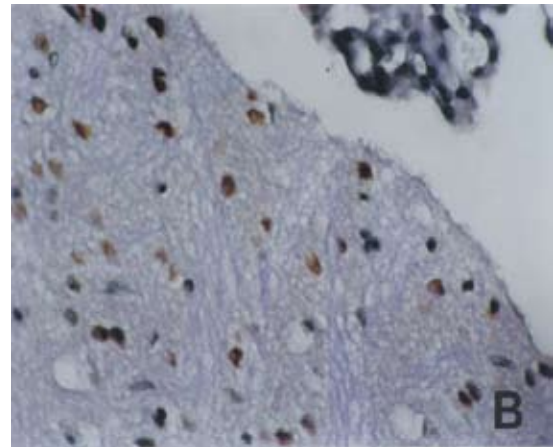
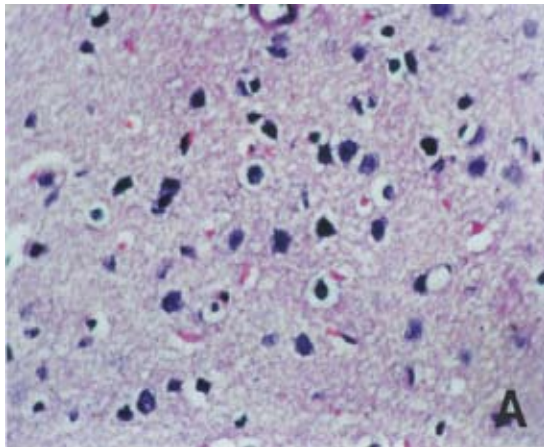
**Results.** Cortical, medullary, and ventricular regions of the brain were examined histologically.

**Control group.** A vascularly rich pia mater surrounding the cortex was seen. In the cortical region, glial cells and euchromatic nucleated neurons with cell processes were observed. In between the neurons and glial cells, neuropil were also observed. Near the ventricular wall of the brain parenchyma, a few apoptotic cells were seen.

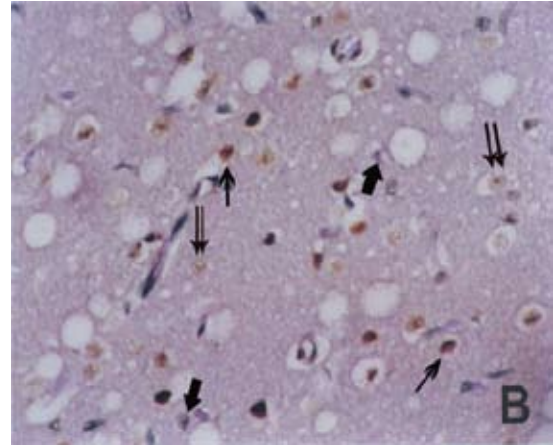
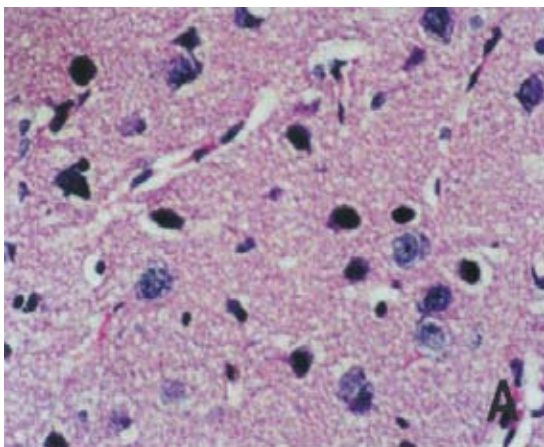
**Toluene group.** There were many pyknotic nucleated cells, most belonged to neurons in the cortex. In addition to the neurons, there were many infiltrative

cells and glial cells (Figure 1a). The cortical parenchyma had very few apoptotic cells, whereas the apoptotic cells were mostly located in the parenchyma near the ventricular wall (Figure 1b). The disturbance of integrity of neuronal processes suggesting an extreme edema in cerebral cortex was observed. Edema between the cells in neuropil was prominent, particularly beneath the pia mater and around the vessels. Almost all blood vessels appeared congested. Pyknotic neurons, in addition to many infiltrative cells in between the neuropil were also observed in the TN group (Figures 1a & 1b).

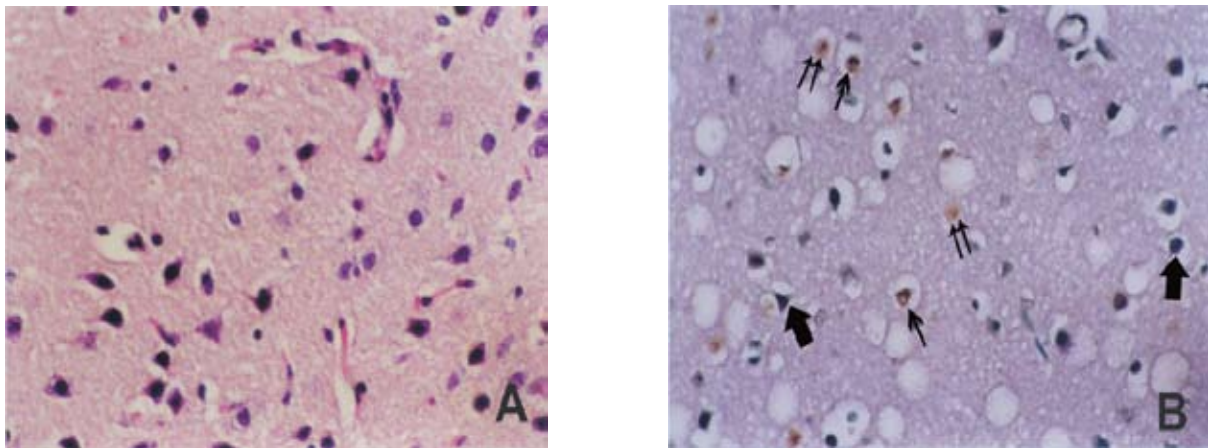
**Toluene + vitamin E group.** In the cortex of the brain, in addition to the euchromatic nucleated neurons, degenerative cells with pyknotic nuclei were observed. Infiltrative cells and glial cells increased. Minimal edema was also present (Figure 2a). The apoptotic cells were more prominent near the ventricular wall (Figure 2b).



**Figure 1** - Histopathological changes of the toluene group showing a) many infiltrative cells and pyknotic neurons (Hematoxylin and eosin x 400.) b) Apoptotic cells near the ventricular wall of brain parenchyma (ApopTag plus peroxidase x 400).



**Figure 2** - Histopathological changes of the toluene + vitamin E group showing a) mild edema, euchromatic nucleated neurons, and glial cells besides degenerative cells (Hematoxylin and eosin x 400). b) Apoptotic (thin arrow), pyknotic (thick arrow) and euchromatic neurons (double arrow) (ApopTag plus peroxidase x 400).



**Figure 3** - Histopathological changes of the vitamin E group showing a) euchromatic nucleated neurons and glial cells in between neuropil and blood vessels (Hematoxylin and eosin x 400). b) A few apoptotic cells (thin arrow) are seen between euchromatic (double arrow) and pyknotic neurons (thick arrow) (ApopTag plus peroxidase x 400).

**Table 1** - The average number of cells in 1 mm<sup>2</sup> area.

Cells	Control	Toluene	Toluene + Vitamin E	Vitamin E
Neurons with euchromatic nuclei	332.1 ± 105.5	106.2 ± 71.3 ( <i>p</i> = 0.001) <sup>*</sup>	373.2 ± 82.0 ( <i>p</i> = 0.000) <sup>†</sup>	424 ± 175 ( <i>p</i> = 0.325) <sup>§</sup>
Pyknotic neurons	170.8 ± 43.0	374 ± 109.2 ( <i>p</i> = 0.002) <sup>*</sup>	298.25 ± 76.0 ( <i>p</i> = 0.128) <sup>‡</sup>	267.2 ± 94.1 ( <i>p</i> = 0.061) <sup>§</sup>
Apoptotic neurons	28 ± 15.3	468 ± 166 ( <i>p</i> = 0.000) <sup>*</sup>	320 ± 125 ( <i>p</i> = 0.033) <sup>†</sup>	216 ± 66.1 ( <i>p</i> = 0.001) <sup>§</sup>
Infiltrative glial cells	148.48 ± 27.83	497.15 ± 124.6 ( <i>p</i> = 0.000) <sup>*</sup>	348.6 ± 44.7 ( <i>p</i> = 0.007) <sup>†</sup>	356.1 ± 48.4 ( <i>p</i> = 0.000) <sup>§</sup>

<sup>\*</sup>*p*<0.005 value of differences between toluene and control values, <sup>†</sup>*p*<0.005, value of differences between toluene + vitamin E group and toluene values, <sup>‡</sup>*p*>0.005 value of differences between toluene + vitamin E and toluene values, <sup>§</sup>*p*<0.005 value of differences between vitamin E and control values

**Table 2** - The mean value ± SD of open field behavioral performance.

Measurement	Control	Toluene	Toluene + Vitamin E	<i>P</i> -value
<i>Crossing</i>	Early response (On the first day)	87.7 ± 22.5	91.7 ± 32.6	0.056
	Late response (On the fifth day)	91.4 ± 22.8 <sup>a</sup>	26.0 ± 16.6 <sup>b</sup>	0.000
	Significance	0.802	0.002	0.001
<i>Center cross</i>	Early response (On the first day)	4.7 ± 2.9	3.9 ± 5.9	0.621
	Late response (On the fifth day)	4.7 ± 3.6	1.2 ± 2.0	0.072
	Significance	1.000	0.402	0.127
<i>Freezing</i>	Early response (On the first day)	3.8 ± 2.8 <sup>a</sup>	15.4 ± 4.8 <sup>b</sup>	0.000
	Late response (On the fifth day)	4.0 ± 1.8	24.5 ± 14.6	0.004
	Significance	0.922	0.141	0.006

<sup>a,b</sup>-Means within a row followed by the same superscript are not significantly different *p*>0.005, (n) mean ± SD

**Vitamin E group.** The histological appearances of the cortex of brains were similar to the control group. In most of the animals no edema was present, whereas some had mild edema in the neuropil. The cortex was rich in euchromatic nucleated neurons and most of the vessels were not congested (Figure 3a). Though the cortical parenchyma lacked apoptotic cells, in the neighborhood of the ventricular wall, apoptotic cells were found moderately (Figure 3b).

**Statistical analysis.** The number of neurons with euchromatic nuclei, pyknotic, and apoptotic neurons, and infiltrative glial cells are shown in Table 1. There was a significant difference ( $p < 0.005$ ) between C and TN groups in the number of euchromatic, pyknotic, and apoptotic neurons, and glial cells. There was also a significant difference ( $p < 0.005$ ) between TN and TN + Vit E groups in the number of euchromatic, pyknotic, and apoptotic neurons, and glial cells. Between the C and Vit E groups, there was a significant difference ( $p < 0.005$ ) between the number of glial cells and apoptotic neurons. However, there was no significant difference ( $p > 0.005$ ) in the number of pyknotic neurons and neurons with euchromatic nuclei.

**Behavioral performance.** Significant differences were found in the open-field test (Table 2). There was a trend for TN + Vit E rats to cross more lines than the toluene and control rats during the entire 5 minute open-field test on day one, but this effect narrowly missed statistical significance ( $p = 0.083$ ). In the TN + Vit E and TN groups at the 5th day of the intervention, the mean values of lines crossed were less than the first day, whereas in the control group these values did not change during the intervention. There were no significant differences among the groups for center-cross measures. In the TN group, on day one ( $p = 0.000$ ) and day 5 ( $p = 0.005$ ), the mean percentage of freezing was significantly greater than the control rats. In the TN group on day one ( $p = 0.000$ ), the mean percentage of freezing was significantly greater than the TN + Vit E group rats. In the TN + Vit E group, on the 5th day of the intervention, the mean percentage of freezing was greater than the first day ( $p = 0.006$ ), whereas in the control and toluene groups these values did not change during the intervention.

**Plasma MDA levels.** The plasma MDA level was significantly higher in the toluene group than in the control group ( $p < 0.005$ ). Treatment with vitamin E significantly reversed the elevations in MDA levels in the TN + Vit E group ( $p < 0.005$ ).

**Discussion.** Toluene is one of the most volatile abused solvents, with a similar pharmacological profile to others, for example, ethanol, barbiturates, and benzodiazepines.<sup>23</sup> It is a worldwide problem as it causes serious behavioral problems in users.

Our histopathological observations concerning the pyknotic cells, namely, cell death and the significant increase of the TUNEL positive apoptotic cells in brain parenchyma, particularly in the TN group, might be related to reactive oxygen species (ROS) formation. One of the important pathways of toluene neurotoxicity is thought to involve the generation of ROS, which induces oxidative damage to lipids, proteins, and nucleic acids that affect normal cell physiology and eventually leads to neuronal demise. The ROS modulate key protein kinases that are involved in signalling pathways that lead to cell death and survival.<sup>24</sup> It has been shown that neurons are remarkably sensitive to nitric oxide (NO), and undergo cell death once exposed to sustained NO generation.<sup>25</sup> Several findings suggest that NO induces apoptosis by regulating pro- and anti-apoptotic protein levels. In this study, the histopathological finding related to the increase of infiltrative glial cells is another important change to be considered. The ROS are reported to act as pro-inflammatory mediators that may explain the increased number of inflammatory cells and glial cells in brain parenchyma. The ROS-stimulated cytokine release potentially accelerates inflammatory conditions.<sup>26</sup> In this study, periventricular recruitment of microglia was thought to result from migration to this site rather than transformation of macrophages into microglia cells that may be exhibited more after a period of time. Carnobell et al<sup>26</sup> showed that microglia migrate long distances into the brain parenchyma towards effected regions in vivo and in situ, in response to many chemokines and growth factors present in the brain.<sup>26</sup>

Edema in the neuropil was another prevalent change in brain tissue of the toluene group observed in our study. The cerebral edema may depend on the breakdown condition of the blood-brain barrier, which causes the accumulation of tissue fluid in the nervous tissue. The breakdown of the blood-brain barrier is suggested to be related not only to the capillary endothelial lining, but also the end-feet of astrocytes located external to the vascular basal lamina. Therefore, the edema was also a result of the disruption of maintenance of fluid transport and ions from the perineural extracellular space to blood vessels. Although in our study the vitamin E only treated group also showed edematous and mild apoptotic changes in the parenchyma, particularly close to ventricular wall, the statistical analysis indicates no significant difference with the control group. The histopathological findings related to edema imply that toluene effects not only the blood circulation, but also may be effective through cerebrospinal fluid circulation, as confirmed by Ameno et al.<sup>27</sup>

With regard to lipid peroxidation, in our study, the analysis of MDA levels was significantly higher in the TN treated group compared with the Vit E, and TN +

Vit E treated groups ( $p=0.001$ ). Biochemical changes in MDA levels confirms the cellular injury of toluene. Many oxygenated compounds, particularly aldehydes such as MDA, are produced during attacks of free radicals on membrane lipoproteins and polyunsaturated fatty acids. An MDA test is the most widely used assay for the measurement of lipid peroxidation.<sup>28</sup> Exposure to toluene, both in vivo and in vitro, leads to ROS formation in many tissues including brain tissue.<sup>29</sup> Nakahashi et al<sup>30</sup> supports our conclusion that vitamin E prevents ROS production by reducing oxidative stress.

The results of our study suggest a more complicated relationship between toluene exposure and behavioral effects. Toluene increased rate of freezing on day one, but had no effect on crossing and center-cross. Toluene increased rate of freezing and decreased crossing on day 5, but had no effect on center-cross. Our data from the open field test suggest that the negative behavioral effects of toluene can be prevented with vitamin E in the early exposure period. An increase in the infiltrative cells in the brain parenchyma has an important role in tissue damage. The activated infiltrative cells release key mediators as toxic oxygen products, proteases, and elastases, resulting in tissue injury. The observed differences in time patterns of behavioral effects point to differentiated mechanisms of action of toluene in the brain.<sup>31</sup> Vitamin E treatment accompanying toluene intake will also reduce the degenerative changes of brain parenchyma by oxidative stresses produced by toluene intake. Recent in vitro studies show a variety of effects of toluene and other solvents on different receptors,<sup>32,33</sup> and calcium channels,<sup>34</sup> which support the idea that solvents may change nervous system function by different mechanisms, which may become apparent in different patterns of behavioral effects. Knowledge of the basis of this neurobehavioral changes is not well known. In our study, when toluene treatment was accompanied vitamin E, this repaired some of the negative behavioral effects (decrease of crossing, increase of freezing) of toluene on day one, but had no effect on day 5. The results of this study show that the behavioral effects of toluene may largely depend on the exposure pattern, and different relationships exist between different behavioral effects and exposure. Toluene abusers reported with marked cortical and functional impairment in neuropsychological test performances, besides neurological impairment. The behavioral effects of toluene are thought to be directly related to internal concentration in the brain, or the total dose administered.<sup>35,36</sup>

Studies on the effects of these abused inhalants are limited in the literature. Lipid peroxidation caused by free radicals is one of the most important pathways in

the pathogenesis of brain damage. Besides biochemical changes, the terms of histopathological changes in the brain may also become apparent by the behavioral alterations. The combination of MDA levels and histopathological changes in our study reveals important oxidative changes to the cells. Vitamin E is present in the lipid bilayer of biological membranes and may play a structural role in many biological systems.<sup>37</sup> Vitamin E provides homeostasis in living cells by a mechanism of incorporation into cell membranes or by entering the cells, and provides the stability of the membrane, particularly cell membranes, which is very important for cell survival. The  $\alpha$ -tocopherol binding protein, which is specific for vitamin E, appears to be related to its inhibitory effect on the autooxidation of unsaturated fatty acids. A broad-spectrum antioxidant, vitamin E, plays an important role in the protection process against endogenous and exogenous oxidative destruction of the cell, especially in the brain. Therefore, many studies on the antioxidant action of  $\alpha$ -tocopherol, vitamin E, or both, suggested that they reduced ROS.<sup>30</sup> It is shown that  $\alpha$ -tocopherol has a marked protective effect on the maintenance of the integrity of membrane complexes of cells, as also suggested by our findings.<sup>38</sup> Additionally, the possibility of a vitamin E benefit on behavioral changes in toluene intoxication, should be a helpful assumption for the social problems of abusers that have not been discussed before in the literature. Those behavioral changes in vitamin E treated groups may be evidence of decreased oxidative injury, which should be tested by an appropriately designed clinical trial.

In conclusion, it is expected that widespread abuse of thinner inhalation among low-income teenagers will present many societies with the need to face a major health problem in the future. The toluene affect seen in brain tissues is a combination of a direct toxic effect and an indirect consequential effect on glial and inflammatory cells by pro-inflammatory mediators and the toxic oxygen radicals they generate. Our results indicate that vitamin E cannot totally antagonize the histopathological degenerative effects of toluene on brain tissue, but has a particular effect on apoptosis of neurons and inflammation of the cells. Our results show that vitamin E expressed a protective role against the toxic influence of toluene on all examined parameters in the rat brain. The biochemical, histological, and behavioral results should be supported by further detailed studies.

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