Vitamin E can compensate the density of M1 receptors in the hippocampus of scopolamine-treated rats

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Abstract
M1 muscarinic receptor plays a fundamental role in memory and is closely associated with Alzheimer's disease (AD); it has long been assumed as a therapeutic goal. But effects of vitamin E on density of M1 muscarinic receptor-immunoreactive (ir) neurons remain poorly understood. The present research aimed to examine the chronic administration effect of vitamin E against scopolamine-induced memory loss and the number of M1 muscarinic receptor-ir neurons of the hippocampus in male rats.

Randomly, 42 adult male Wistar rats were divided to six groups: control, Sham-saline: receiving scopolamine + saline, Sham-sesame oil: receiving scopolamine + sesame oil and three experimental groups: receiving scopolamine + vitamin E with different doses (25, 50, and 100 mg/kg/day, i.p.) for 14 days. The passive avoidance task was used for the memory test. Twenty-four hours after behavioral tests, rats’ brains were taken and fixed, and after tissue processing, sections were stained using the immunohistochemical technique for M1 muscarinic receptor-ir neurons and cresyl violet for neurons.

The injection of scopolamine to rats caused memory impairment and vitamin E treatment could ameliorate it. In the scopolamine-treated groups, the number of CA1 and CA3 pyramidal and dentate gyrus (DG) granular neurons was decreased significantly as compared to the control group. Vitamin E treatment significantly increased neuron numbers in the CA1 and CA3 areas of the hippocampus and DG area. Treatment with vitamin E for 14 days could compensate the loss of M1 muscarinic receptor-immunoreactive neuron numbers induced by scopolamine in the hippocampus. The most effective vitamin E dose was 50 mg/kg/day in this study.

In conclusion, vitamin E can compensate the neuronal loss in the hippocampal formation and also it can raise the density of M1 receptor-ir muscarinic neurons after an injection of scopolamine.

Key words: vitamin E, scopolamine, M1 muscarinic receptor, hippocampus, passive avoidance memory test.

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Introduction

One kind of G-protein coupled receptors is muscarinic acetylcholine receptors [16,68,95]. So far, five muscarinic acetylcholine receptor subtypes (M1-M5) have been known [13,14,27] and among them, the M1 subtype makes up more than a half of the total and mainly exists in all major zones of the forebrain, such as the cortex, the hippocampal formation, corpus striatum of basal nuclei, and thalamus [30,36,65]. Indeed, M1 muscarinic receptors are highly concentrated in the brain areas related to Alzheimer’s disease (AD) but less in the periphery [59,60]. Cognitive deficits and damage in long-term potentiation were shown in M1 muscarinic receptor-knockout mice, indicating that this receptor is physiologically related to multiple roles such as neuronal excitability, synaptic plasticity, and differentiation of neurons during early development, and memory [3,41,65,83,84,94,96]. Because M1 muscarinic receptor plays a critical role in memory and is closely linked with AD, it has long been guessed as a target for therapy [28].

Previous studies reported the precognitive effects of M1 muscarinic receptor activators and have used models in which the cholinergic function is damaged with scopolamine, a non-selective muscarinic receptor antagonist [78]. Scopolamine is well known for interfering with the methods of learning acquisition, memory performance and short-term memory in animals and humans [32,47,63]. The post-training scopolamine dose-dependently decreased the step-through latency in the inhibitory avoidance task; it shows scopolamine-induced amnesia [42]. Also, scopolamine reduced dose-dependently the number of M1 muscarinic receptor-immunoreactive (ir) neurons in the male rats’ hippocampus [44]. Moreover, scopolamine directly caused damage to the hippocampal circuits that might predominantly be responsible for cognitive and memory deficits [18]. The chronic systemic treatment with scopolamine significantly disrupted cell proliferation, differentiation and maturation, especially, impaired the dendrite maturation and complexity of neuronal progenitor cells in the mouse hippocampal dentate gyrus (DG) [100]. A common way for a large number of degenerative routes in AD is the neuronal loss [87,93] and may be prompted by some factors, such as perturbed calcium regulation, inflammatory routes or oxidative stress, ischemia, amyloid-\(\beta\) plaques and glutamate [8,19,72]. Additionally, most researches have concentrated broadly on amyloid-\(\beta\) deposits and neurofibrillary pathology in AD, while neuronal loss has been more difficult to assess [86]. In the AD hippocampus, the neuronal loss can describe the memory disorders which are clinical signs, even in the preclinical stages [53]. The loss of neurons is commonly prominent in the hippocampus, mainly the CA1 region, and is further noticed throughout the cerebral cortex, increasing with disease progression [15]. Also, in both AD and normal aging, the distribution of the neuronal loss in the hippocampus is not very well understood [97]. This selective loss of neurons could locally be related to understanding the complicated mechanism of AD [73]. Also, a neuronal loss in the hippocampus in microvascular dementia patients was described in 2002 by Kril et al. [55]. This could have essential suggestions in the design of therapeutic and investigative strategies in AD [73]. Drugs currently used for AD only controlled the symptoms and slow the progression of the cognitive decline. There is no effective treatment to delay or stop the progressive brain damage [22].

Numerous studies have documented increased oxidative stress in the plasma and cerebro-spinal fluid of AD patients, which can be observed as an increase in lipid peroxidation [9,80]. Therefore, antioxidants, such as vitamin E, have frequently been discussed as a potential therapeutic option in AD [5,58].

Health benefits of vitamin E include antioxidant, neuroprotective, and anti-inflammatory properties [70]. Vitamin E can reduce or prevent memory deficiencies that accompany several disorders for example mental stress [67], ischemic injury of cerebrum [1], AD [50,79], stroke [88] and aging [48]. The effect of vitamin E supplements on memory damage has been studied in aged rats, it caused marked retention of their memory function [90]. Moreover, when vitamin E was given to moderately severe AD patients, those patients showed delayed beginning of severe dementia [79]. It has been shown that long-term, high-dose vitamin E supplementation in the elderly significantly develops the cognitive function [34]. Although trials examining the efficacy of vitamin E supplementation in the AD treatment have yielded inconclusive results [25,61,76,79], a combination of \(\alpha\)-tocopherol and inhibitors of angiotensin-converting-enzyme have been newly confirmed as effective in attenuating the cognitive decay in AD.
patients [21]. Taken together, these findings and others [17, 54, 69, 82] determine a crucial role for vitamin E in preserving emotional responses, learning and memory. Importantly, vitamin E has been described to have more interaction with the cholinergic system in processes of memory retention [23].

Since vitamin E decreased scopolamine-induced damage on memory retention; it may act through activation of the cholinergic system on memory retention [23]. However, to the best of our knowledge, there have been no reports on the density of M1 muscarinic neurons that contain receptors in the hippocampus after administration of vitamin E. Therefore, the present study examined the effect of chronic administration of vitamin E on scopolamine-induced AD-like impairment memory and the changes of M1 muscarinic receptor-ir neurons number in the male rat hippocampus.

Material and methods

Animals

Forty-two male adult Wistar rats (8 weeks old; 200 ±20 g) were provided by the Pasteur Institute (Tehran, Iran). The animals were maintained in individual cages with a 12 : 12 hour light and dark cycle (light beginning at 7:00 a.m.) and also they had free access to water and food. The temperature of the animal house was 22 ±3ºC. All experiments were performed during the light phase between 8:00 a.m. and 14:00 p.m. The Ethics Committee in Golestan University of Medical Sciences approved all procedures described in the method. We tried to use the minimum number of rats and we tried to minimize the suffering of animals.

Inhibitory avoidance apparatus

The inhibitory avoidance task, step-through, consisted of the same size (20 × 20 × 30 cm³) light and dark boxes. Between two boxes, a guillotine door (7.9 cm²) could be lifted manually. The floor of the dark box was made by stainless steel bars with 1 cm intervals. An isolated stimulator produced sporadic electric shocks (50 Hz, 3 s, and 1.5 mA intensity) to the grid floor of the dark chamber.

Behavioral procedures

Our previous studies [62, 81] explained passive avoidance memory as follows: for 1 h before the start of the tests, rats were allowed to habituate in the testing room. Then, one rat was placed in the light box; after 5 s, the guillotine door was opened. The animal can enter the dark chamber. The latency was recorded to entrance the dark compartment. After waiting more than 120 s to enter the dark box, this rat was excluded from the experiments.

When all four-paws of the animal entered the next compartment, the guillotine door was closed. This trial was repeated after 30 min. In the acquisition trial, when the animal entered the dark (shock) box, the door was closed. Immediately a foot shock (50 Hz, 1.5 mA and 3 s) was sent to the grid floor of the dark chamber. After this shock, the rat was removed from the Shuttle Box. Two minutes later, the test was repeated and if the rat did not enter the dark box during 120 s, positive acquisition of inhibitory avoidance response was recorded. The rat was backed to the cage, if it learned inhibitory avoidance response successfully.

Each animal on the test day was gently placed in the light box for the retention trial and the latency time to enter the dark box was recorded and termed as step through latency. The retention trial was set a limit of 300 s as cut-off time.

Experimental design

We distributed the rats randomly to the following groups (n = 7):

- Control group: had no any drugs and behavioral tests;
- Scopolamine-saline group: receiving scopolamine (Tocris, UK) with a single dose of 3 mg/kg (i.p.) for a day [45], and then an injection of 0.9% sterile saline (1 ml/kg, i.p.) for fourteen days, and with a behavioral test;
- Scopolamine-sesame oil group: receiving a single dose of scopolamine 3 mg/kg for a day and then receiving sesame oil (1 ml/kg, i.p.) for fourteen days, and with the behavioral test;
- Three scopolamine-vitamin E treated groups: receiving a single dose of scopolamine 3 mg/kg for a day and then an injection of vitamin E (Darou Pakhsh Pharmaceutical Mfg Co., Iran) with different doses (25, 50, and 100 mg/kg/day, i.p.) [7, 23, 38] for fourteen days, and with the behavioral test.

Scopolamine, a muscarinic receptor antagonist, was dissolved in 0.9% sterile saline and vitamin E
was dissolved in sesame oil. Twenty-four hours after the scopolamine injection and the last injection of drugs, the rats were tested for the retention trial in inhibitory avoidance apparatus.

**Perfusion and sectioning**

Twenty-four hours after the end of the behavioral test, the rats were transcardially perfused with normal saline and then with 4% paraformaldehyde solution (Scharlau, Spain). The brains were removed and fixed in 4% paraformaldehyde for a week. After dehydration and clarification with xylene, the paraffin blocks of brains were prepared. Coronal serial sections (6-µm thick) of the brain with an interval of 20 µm were processed for immunohistochemical and cresyl violet staining [66].

**Immunohistochemical staining**

The process of staining with the antibody against M1 muscarinic receptor was as follows [44]:
1. Incubation of the brain slices at 37°C for 30 minutes.
2. Deparaffinization and hydration of slices embedded in xylene and a graded series of ethanol.
3. Washing with distilled water.
4. After incubation at 60°C for 5 minutes, the sections were covered with an epitope retrieval solution (IHC World, USA) at 90°C for 15 minutes.
5. For cooling, they were endorsed for 20 minutes at room temperature.
6. Washing with washing buffer (PBS/Tween 20 in 0.1% Triton X-100).
7. For 10 minutes at room temperature, the peroxidase blocking solution (IHC World, USA) was used.
8. For 30 minutes at room temperature, the slices were incubated with the avidin/biotin blocking solution (IHC World, USA) and rinsed with PBS.
9. For 60 minutes at 37°C, sections were covered with the Anti-Muscarinic Acetylcholine Receptor 1 Rabbit polyclonal antibody (1 : 200, Abcam Inc., USA) and then the washing buffer.
10. After this step, slices must be incubated for 60 minutes with immunoglobulin G (IgG) (Abcam Inc., USA) at 37°C and washed with the washing buffer.
11. Incubation with Streptavidin HRP protein (1 : 5000, Abcam Inc., USA) at room temperature for 30 minutes and the washing buffer.
12. By using DAB (Dako, Denmark), the M1 muscarinic receptors were visualized.
13. Finally, the brain slices were cover-slipped with entellan (Merck, Germany).

**Cresyl violet staining**

The brain slices were deparaffinized in xylene and hydrated with ethanol and washed with distilled water. Then, the sections were stained for 5 min in 0.02% cresyl violet (Sigma, USA) solution and washed quickly in distilled water. Finally, the slices were cover-slipped with entellan [81].

**Image processing and cell counting**

Using a BX51 light microscope (Olympus, Japan) and DP 72 digital camera (Olympus, Japan), images were taken. 40× magnification for hippocampal CA1 and CA3 areas (30 000 µm²) and 100× magnifications for DG area (4800 µm²) in all sections were selected randomly. To count the number of M1 muscarinic receptor-ir neurons in the hippocampus, OLYSIA Autobioreport software (Olympus, Japan) was used, the M1 muscarinic receptor-ir neurons were counted manually [43,46,49] and counting was performed blind to treatment.

**Statistical analysis**

All of our data were expressed as mean ± SD. SPSS v.16 (Armonk, NY, USA) was used for statistical analysis. For normal distribution of data, the Shapiro-Wilk test was approved for the statistical evaluation. We analyzed the data with the one-way analysis of variance (ANOVA) followed by post-hoc LSD (least significance difference) test for over-all various comparisons between groups and p < 0.05 was considered to be statistically significant.

**Results**

During the memory retention test, the latency to enter the dark box was reduced after scopolamine treatment compared to the training day, indicating memory impairment (Fig. 1). Vitamin E administration (25, 50, and 100 mg/kg/day) increased significantly the step-through time of latency when compared to the scopolamine-saline group (p < 0.01,
vitamin E and M1 muscarinic receptors

There is a significant difference in step-through latency time between the 50 mg/kg/day dose of vitamin E compared to the 25 mg/kg/day dose of vitamin E ($p < 0.001$, Fig. 1). These results reveal that vitamin E, only at an intermediate dose (50 mg/kg/day), inhibited the harmful effects of scopolamine.

In Figure 2, coronal sections of the hippocampus for CA1 area stained by immunohistochemistry anti-M1 muscarinic receptor was shown.

Furthermore, Figure 3 shows the number of neurons contained in the M1 muscarinic receptor-ir in the rat hippocampus. An injection of scopolamine caused the hippocampal M1 muscarinic receptor-ir neuron loss in the CA1, CA3 and dentate gyrus. Comparison of the mean number of M1 muscarinic receptor-ir neurons in control and scopolamine-saline groups of rats revealed that scopolamine significantly reduced the number of M1 muscarinic receptors in different areas of the hippocampus ($p < 0.001$, Figs. 3A and B).

In the scopolamine-saline group, the mean number of neurons with the M1 muscarinic receptors in the CA1 area was $25.52 \pm 9.05$, and in the CA3 area it was $20.30 \pm 5.54$, respectively.

We found that vitamin E significantly raised the amount of M1 muscarinic receptor-ir neurons in all areas of the hippocampus compared to the scopolamine-saline group (Figs. 3A-C). According to our findings, a 50 mg/kg/day dose of vitamin E appears to attenuate the scopolamine-induced M1 muscarinic receptor-ir neuron loss in all areas of the hippocampus (Figs. 3A-C). The higher mean number of M1 muscarinic receptor-ir neurons for the vitamin E-treated group with a dose of 50 mg/kg/day was $38.12 \pm 14.54$ in the CA1 area of the hippocampus (Fig. 3C).

Figure 4 shows a representative cresyl violet-stained coronal section of the hippocampal CA1 area. In the scopolamine-treated groups, CA1 and CA3 pyramidal neuron numbers ($25.62 \pm 7.27$, $24.60 \pm 8.36$ respectively) were significantly decreased as compared to the control group ($40.20 \pm 13.52$, $31.82 \pm 7.79$, respectively) (Figs. 5A, B, $p < 0.001$). Indeed, an intraperitoneal injection of scopolamine caused the hippocampal cell loss.

Vitamin E treatment significantly increased the number of pyramidal neurons in the CA1 and CA3 areas. Comparison of the mean number of neurons in vitamin E-treated groups revealed that vitamin E (50 mg/kg/day dose) has a significant neuroprotective effect on the scopolamine-induced neuron loss (Figs. 5A, B). The mean number of neurons in CA1 and CA3 for the vitamin E-treated group (with a dose of 50 mg/kg/day) was $38.80 \pm 8.14$ and $31.20 \pm 8.39$, respectively.

The mean number of DG granular neurons ($19.42 \pm 4.86$) after scopolamine treatment was significantly decreased (Fig. 5C, $p < 0.001$) as compared to the control group ($36.15 \pm 12.01$). The comparison between scopolamine-saline and vitamin E-treated groups revealed that vitamin E increases significantly

Fig. 1. The time of latency (per second) to enter the dark box. The rats under different groups were administered with an equivalent volume of scopolamine (3 mg/kg, i.p.) for a day and then saline, sesame oil and vitamin E (25, 50 and 100 mg/kg/day, i.p.) was given to all the groups for fourteen days. The step through latency time was 120 s for all the groups. Twenty four hours after the scopolamine injection (base line) and after the last injection of drugs, the rats were tested for the retention trial in the inhibitory avoidance apparatus. Data represent means ± SD from 7 rats ($n = 7$) in each group. **$p < 0.01$ and ***$p < 0.001$ differences from the Sco-Saline group, $$$p < 0.001$ difference from the Sco-Vit E (50 mg/kg/day) treated group.
Fig. 2. Coronal sections of CA1, CA3 areas of the rat hippocampus and DG stained by immunohistochemistry against M1 muscarinic receptor, from control, scopolamine-saline, scopolamine-sesame oil, scopolamine – 25 mg/kg/day vitamin E, scopolamine – 50 mg/kg/day vitamin E, scopolamine – 100 mg/kg/day vitamin E groups. Scale bars = 20 µm. Arrows demarcate M1 muscarinic receptor-immunoreactive neuron in the hippocampal CA1, CA3 and DG areas of all groups.
Fig. 3. The M1 muscarinic receptor-ir neuron numbers in the hippocampal CA1, CA3 and DG areas. Data represent means ± SD. ##p < 0.01 and ###p < 0.001 differences from the control group, **p < 0.01 and ***p < 0.001 differences from the Sco-Saline group, $p < 0.05$ and $$p < 0.01$ differences from the Sco-Vit E (50 mg/kg/day) treated group.
CA1    | CA3    | DG

Control

Scopolamine-Saline

Scopolamine-Sesame oil

Scopolamine-Vitamin E 25 mg/kg/day

Scopolamine-Vitamin E 50 mg/kg/day

Scopolamine-Vitamin E 100 mg/kg/day

Fig. 4. Neurons in the CA1, CA3 and DG areas of the hippocampus stained by cresyl violet (neurons are purple) from the control, scopolamine-saline, scopolamine-sesame oil, scopolamine – 25 mg/kg/day vitamin E, scopolamine – 50 mg/kg/day vitamin E, scopolamine – 100 mg/kg/day vitamin E groups. Scale bars = 20 μm.
Fig. 5. The effects of vitamin E on the hippocampal neuron numbers in scopolamine-treated rats. Data represent means ± SD. ###p < 0.001 difference from the control group, *p < 0.01 and ***p < 0.001 differences from the Sco-Saline group, $p < 0.05 and $$p < 0.01 differences from the Sco-Vit E (50 mg/kg/day) treated group.
the scopolamine-induced neuron reduction (Fig. 5C, \( p < 0.001 \)). The most effective dose of vitamin E was 50 mg/kg/day and it protects hippocampal DG granular neurons against scopolamine. The mean number of granular neurons for the vitamin E-treated group with a dose of 50 mg/kg/day was 34.15 ±9.42 neurons.

**Discussion**

The present study suggested that vitamin E could increase M1 muscarinic receptor-ir neuron density in the hippocampus of scopolamine-treated rats. Also, vitamin E treatment could improve the scopolamine-induced neuronal loss and memory impairment. Vitamin E seems to have a significant neuroprotective effect on scopolamine.

In this study, we found that a single dose of scopolamine, as an antagonist of muscarinic receptors, could impair passive avoidance memory. Similarly, several lines of evidence have shown that scopolamine can cause a very potent impairment on tests of memory [20,37,40]. Moreover, an intrahippocampal [6] or intra-peritoneal [56,81] injection of scopolamine impairs the passive avoidance memory. Some previous studies indicate that both subtypes, M1 and M2, of muscarinic receptors were important for memory association of inhibitory avoidance [77].

In the present study, vitamin E treatment significantly improves the passive avoidance memory. Consistently with our findings, some studies demonstrated that vitamin E potentiated memory retention [23,38,51]. Also vitamin E has been reported to avoid the aging-induced memory deficits [29,90]. It has been reported that vitamin E with activation of the cholinergic system could help the memory maintenance [23].

According to our findings, previous research confirms that a scopolamine injection causes cell loss in hippocampal neurons [81]. Also, another study has reported a significant loss of hippocampal neurons especially in both CA1 and CA3 areas in AD [73]. Besides, previous studies have shown that neuronal loss has occurred in many mouse models of AD [10,12,39,99] and AD patients [98].

Also, we found that vitamin E can increase hippocampal pyramidal and granular neuron numbers after the scopolamine injection. Vitamin E can cause delay or inhibit a clinical diagnosis of AD in elderly people with mild cognitive impairment [35]. Also, Nishida et al. indicated that chronic lipid peroxidation due to vitamin E depletion enhances the AD phenotype in a mouse model [71]. Moreover, memory weakening was slowed in moderately severe AD patients when they took vitamin E supplements [2]. Furthermore, it has been shown that long-term high dose vitamin E supplementation in the elderly patients significantly increases the cognitive function [34].

Consistently with earlier findings [44], our results revealed that treatment of Wistar rats with scopolamine led to decrease M1 muscarinic receptor-ir neuron numbers in the hippocampus. Similarly, after an injection of scopolamine to dogs, the older dogs showed a significant decrease in the density of the muscarinic receptor in some areas of cortex [4]. Similarly to our study, Araujo et al. found a decrease in muscarinic neurons. Furthermore, the M1 immunoreactivity was markedly decreased in AD brains [85] and also an age-related decrease in the M1 receptor has been reported [91]. Some other researches confirm a scopolamine injection, severe cell losses in hippocampal cholinergic neurons [44,57].

The present study showed that vitamin E treatment increases the hippocampal M1 muscarinic receptor-ir neuron numbers in scopolamine-treated rats. Recent studies have shown many useful health effects of vitamin E such as antioxidant and anti-inflammatory properties [52]. Many studies reported that vitamin E can act as an antioxidant in opposition to oxidative factors [74,75,89]. Some studies have revealed that vitamin E can decrease the levels of brain lipid peroxidation and protects it against neuronal damage [11,64,92]. Similarly, we have found that vitamin E compensates the reduction of M1 muscarinic receptor-ir neuron numbers in scopolamine-treated rats. Nevertheless, there are a few studies along the mechanisms to describe the effects of vitamin E. Moreover, vitamin E could have different roles distant from being an antioxidant in cellular mechanisms [24].

The role of vitamin E in protection against AD pathology has also described [31]. Experiments in vitro and in vivo confirmed a mechanism of vitamin E protection against the formation of the hyperphosphorylated tau. In this case, vitamin E was able to inhibit the activation of p 38 mitogen-activated protein kinases, whose activity is critical for the phosphorylation of neuronal tau molecules [31]. Therefore, further research is required to verify the
evidence that vitamin E as a nutritional compound can endorse healthy brain ageing and helps to delay the AD-related functional decline [26]. Antioxidative effects of vitamin E, under certain conditions, may also be useful in the brain. However, beside these helpful roles, vitamin E potentially can increase amyloid-β. α-tocopherol which differs among vitamin E types, has the weakest amyloidogenic potency. So, further researches are suggested to explain the potential role of these various vitamin E species with respect to AD and to detect which form has antioxidantic properties without having an amyloidogenic potential [33].

In conclusion, our results reveal that vitamin E can compensate the neuronal loss and it can increase the number of M1 muscarinic receptor-ir neurons in the hippocampus after exposure to scopolamine. Therefore, vitamin E may have a therapeutic significance for AD.

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Disclosure

The authors report no conflict of interest.

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