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Original article

Electrophysiological, Behavioral and Molecular Study of Vitamin E and *Ginkgo biloba* in a Rat Model of Alzheimer's Disease

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Abstract

Background and objectives: Alzheimer's disease (AD) is characterized by progressive cognitive decline. Oxidative stress plays a central role in the pathogenesis of AD. It has been proposed that administration of antioxidants affect cognitive processes, such as learning and memory. This study investigated the protective effects of vitamin E and *Ginkgo biloba* extract (as antioxidants) on learning and memory, hippocampal plasticity, and apoptotic marker proteins in a rat model of AD. **Methods:** The hyroalcoholic extract of *Gingko biloba* leaves was prepared using maceration method. Male Wistar rats were randomly divided into six groups: control, sham received intra-hippocampal injection (I.H.P) of vehicle, AD model that received intra-hippocampal injection of the beta-amyloid (Aβ), AD+ vitamin E (200 mg/kg, i.p.), AD+ *G. biloba* (100 mg/kg/p.o.), and AD+ vitamin E (200 mg/kg, i.p.)+ *G. biloba* (100 mg/kg/p.o.). At the end of the treatments, the rats were subjected to the passive avoidance learning (PAL) test. The field long wterm potentials (LTP) were recorded in the hippocampal dentate gyrus. Hippocampal expressions of Bax and Bcl-2 (as pro-apoptotic, as anti-apoptotic) proteins were measured by western blot method. **Results:** Treatment with *G. biloba* and vitamin E improved the Aβ-induced memory impairment in the PAL task. Vitamin E and/or *G. biloba* extract enhanced the population spike amplitude evoked potentials of the LTP components, vitamin E and/or *G. biloba* extract increased Bcl-2 expression and decreased Bax expression in the hippocampus. **Conclusion:** *Ginkgo biloba* and vitamin E could suppress the expression of apoptosis markers and improved hippocampal LTP impairment and the memory deficit induced by Aβ.

Keywords: *Ginkgo biloba*; hippocampus; long- term potentiation; memory; vitamin E

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Introduction

Alzheimer's disease (AD), as a common age-related dementia, is a neurodegenerative disorder associated with progressive cognitive impairment and synaptic failure [1]. According to estimates, until 2040, about 81.1 million people will have Alzheimer's disease World Health Organization regions [2]. Cholinesterase inhibitors and glutaminergic N-methyl-D-aspartate receptor antagonists are the available treatments for AD. They may temporarily improve cognition, mediate neurotoxicity but do not modify disease progression [3,4]. Finding novel treatments for Alzheimer's disease is important and emphasized by the World Health Organization [5]. It is necessary to know effective factors in the occurrence, prevention or treatment of AD. The

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better understanding about mechanism, action and treatment methods of effective compounds on AD is the interest of researchers.

Extracellular accumulation of beta-amyloid (Aβ), intracellular accumulation of neurofibrillary tangles (NFT) and neuronal loss are the major neuropathological disorders in the brain of patients with AD [6]. These pathological events occur in specific brain areas and the hippocampus is one of the earliest to be affected by AD [6]. The hippocampus, a bilateral, laminal formation located in the middle temporal lobe, plays important roles in learning and memory and in information-storage [6,7]. It has been shown that in rodents accumulation of Aβ in the hippocampus can cause changes in hippocampal long-term potentiation (LTP), leading to learning and memory impairments [8].

LTP, a form of neuroplasticity, is the primary experimental model of synaptic plasticity that occurs during learning and memory [9]. It has been reported that dysregulation of synaptic plasticity underlies a large number of neurodegenerative disorders such as Alzheimer's disease [10]. The lack of neuronal reserve and insufficient neuroplasticity are attractive theories to explain the pathogenesis of AD, with a gradual decline in synaptic function eventually leading to the neuronal loss [11,12]. Several studies have demonstrated that Aβ- induced damage in hippocampus might underlie LTP and impaired cognition and memory [10,11,13].

The aggregation and deposition of Aβ fibrils lead to mitochondrial dysfunction [14] oxidative stress and apoptosis [14]. Various proteins are involved in the regulation of apoptosis. The Bcl-2 protein family, which include proteins that inhibit and promote apoptosis (such as Bax and Bcl2), are key regulators of this process [15] There is a close link between Bcl2/Bax ratio and neurodegenerative diseases [16]. Aβ promotes neuronal death, in the brains of Alzheimer indiced rats [17]. Aβ peptide induces cell death via Bax/bcl-2 ratio increase [18,19]. Bax is a pro-apoptotic protein and Bcl-2 is an anti-apoptotic protein [20].

Vitamin E (α -tocopherol) is a lipid-soluble compound and is exclusively obtained from the diet [25]. Vitamin E is a potent chain-breaking antioxidant that protects cell membrane against oxidative damage [22,23]. Evidence from animal models shows that vitamin E may be able to counteract oxidative stress induced by Aβ and delay memory deficits [24,25]. Vitamin E-deficiency causes impairment of long-term potentiation in rats [26]. Vitamin E is involved in the hippocampus long-term potentiation [26].

Recently, herbal therapy has been tested for preventing the onset or progression of dementia [27]. *Gingko biloba* extract has been applied as a traditional Chinese medicine for numerous years [28]. *G. biloba* L. extract consists of 24% flavonol glycosides (e.g. kaempferol, quercetin, and isorhamnetin derivatives) and 6% terpene trilactones (e.g. ginkgolides A, B, C, J and bilobalide) [29]. Numerous studies have verified that *G. biloba* has a combination of antioxidative and antiapoptotic effects [27]. *Ginkgo biloba* augments endogenous antioxidant enzymes, and reduces acetylcholinesterase activity in the scopolamine rat model [30]. It is associated with more cognitive benefits in AD under cholinesterase inhibitors treatment [27] and decreases Aβ caused apoptosis in hippocampus neurons [27,31] .

The purpose of this study was to investigate the protective effects of vitamin E and *G. biloba* extract alone and in combination on memory and LTP. Also, the effect of vitamin E and *G. biloba* extract was investigated on expression of Bax and Bcl-2 proteins (as markers of neuronal apoptosis) in a rat model of Alzheimer's disease.

Materials and Methods

Ethical considerations

All experimental procedures were approved by the research and ethics committees (ethical code: 6.35.9.134, Hamadan university of medical science, Hamadan, (Iran) and were performed according to the Guide for Care and Use of Laboratory Animals published by the United States National Institutes of Health (NIH Publication No. 85-23, revised 1985).

Chemicals

Vitamin E and normal saline were purchased from Osveh and Shahid Ghazi Companies (Iran), respectively. Aβ-protein fragment (25-35), urethane, anti-Bcl-2 and anti-Bax antibodies were obtained from Sigma-Aldrich Company (USA). ECL Western Blotting Substrate Kit, horseradish peroxidase, β-actin antibody were purchased the Abcam Company (USA). Nitrocellulose membrane was provided from Amersham Pharmacia Biotech Company (UK).

Plant material

Ginkgo biloba leaves were obtained from the medicine herb garden of Noshahr (Iran) and identified by Dr. Rafieian and were kept under the herbarium Voucher No. 1013 at Medicinal Plant Research Center of Shahrkord University of Medical Sciences. Then, dried in shadow at room temperature. The extraction was performed by maceration method. The dried plant was ground in to a powder. Next, 200 g of the powder was added to 2000 mL of 70% ethanol.in dark at room temperature. After placing the mixture for 72 hours at room temperature with occasionally shaking, it was filtered, and subjected to evaporation and concentration using a rotary evaporator at 4 °C. Then the concentrate was dried at the laboratory temperature and, dissolved in saline when used.

The rats received 100 mg/kg of *G. biloba* hydroalcoholic once daily for 10 consecutive days [32-34]. The dosage was selected from previous studies [32-35].

Aβ peptide preparations

This study used the fragment Aβ peptide because the toxic effects of complete Aβ peptide has been demonstrated before [13,36]. The Aβ peptide was prepared in the following method to minimize the formation of beta-sheet content as reported previously [37]. Aβ (25-35) peptide was solubilized in dimethyl sulfoxide and normal saline (0.9%). Then, the aliquots of Aβ (25-35) solution was preserved at -70 °C until use. After two weeks for AD induction with Aβ peptide [38], the agent treatments was performed for 10 consecutive days. At the end of treatments, passive avoidance learning (PAL) was assessed in two consecutive days.

Animals

Fifty-four male adult Wistar rats weighing 260-300 g were used (Pasteur Institute, Tehran, Iran). The rats were maintained at a temperature of 20 ± 2°C and relative humidity of 50 ± 5% on a 12-h light/dark cycle. The animals were housed in cages with 3-4 rats in each cage with free access to food and water.

Study design

Fifty-four male rats were divided into six following experimental groups (n = 9/ group). Control non-operated received saline (0.9%) orally by gavage for 10 consecutive days. Sham-operated group (received single bilateral intrahippocampal injection of 6 µL sterile saline in the stereotaxic surgery and then spent two weeks recovery, after that gavage saline for 10 days. AD model group received 6 µL Aβ intrahippocampal in the stereotaxic surgery and after two weeks recovery received saline orally for 10 consecutive days. AD+vitamin E group received 6 µL Aβ intrahippocampal in the stereotaxic surgery and after recovery received vitamin E (200 mg/kg) orally by gavage for 10 consecutive days [39]. AD+Gb group received 6 µl Aβ intrahippocampal in the stereotaxic surgery and after two weeks recovery received *G. biloba* (100 mg/kg) orally by gavage for 10 consecutive days. AD+Vit E+Gb group received 6 µL Aβ I.H.P in the stereotaxic surgery and after two weeks recovery received both vitamin E and *G. biloba* for 10 days (Figure 1).

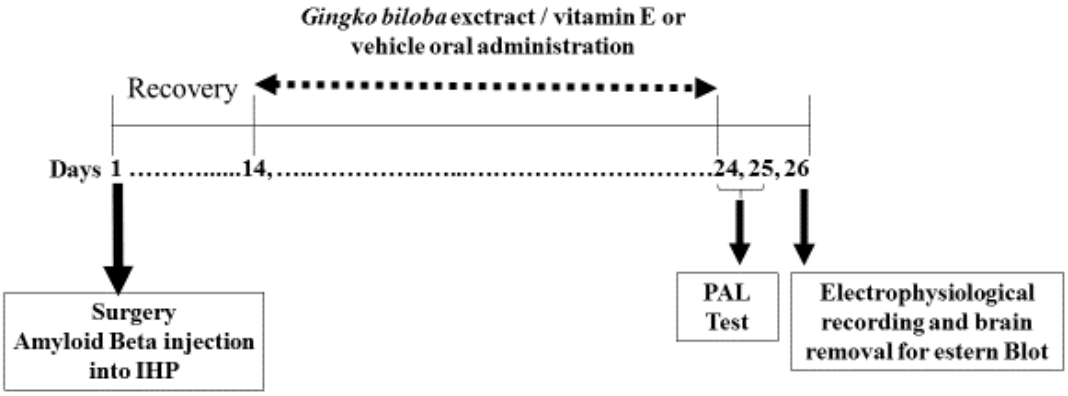

Figure 1. Experimental timeline

Surgery and inducing AD model

The animals were anesthetized with intraperitoneal (i.p.) injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Anesthetized rats were transferred to the stereotaxic apparatus (Stoelting, USA) and the scalp cut retracted. The head position adjusted to place bregma and lambda in the same horizontal plane. Injections were made using a 10 μL microsyringe (Hamilton, USA). Relative to bregma, the coordinates for the dentate gyrus were posterior -3.8, lateral ±2.4, and dorsal 2.7 mm [40]. Aβ solution (6 μL) was bilaterally injected into the region at 1 μL/2 min. Following injection, the microsyringe left in place for 2 min to allow diffusion of the solution. Sham-operated rats received the same volume of vehicle (sterile saline). Following injections, the skin was sutured and the animals were allowed to recover in a warm box before returned to their home cages.

Passive avoidance learning (PAL) test

Apparatus

The passive avoidance test is used to evaluate learning and memory performance. The apparatus and procedure were the same as described in previous studies [41]. Briefly, the step-through passive avoidance apparatus consisted of two compartments: bright and dark (20×20×30 cm). These chambers connected through a guillotine door. The floor of both chambers consisted of stainless steel rods (3 mm diameter) and connected to a shock stimulator. Electrical shock (50 Hz, 0.4 mA, 1.5 s) transferred by an insulated stimulator to the grid floor of the dark compartment.

Habituation

All rats were habituated to the experiment room 30 min before the experiments. First, all experimental groups habituated to the apparatus during two trials. For these trials, each rat was placed in the light compartment and 10 s later, the door raised. Rats have a natural preference for the dark environment. Once the rat entered the dark compartment, the door closed and after 30 s, the rats were taken from the dark compartment and placed in their home cage. The habituation trial was repeated after 30 min and followed the same interval by the first acquisition trial. The second habituation trial was repeated 30 min later.

Training

In the training trial, when the rats had spontaneously entered the dark compartment, the door closed and an electrical shock was applied. After 20 s, the rat returned to its home cage. The latency to entering the dark compartment (step-through latency during acquisition, STLa) was recorded when the animal had placed all four paws in the dark compartment. After 2 min, the procedure was repeated. The training terminated when the rat remained in the light chamber for 120 consecutive seconds. The number of trials to achieve acquisition (a refusal to enter into the dark chamber) was recorded.

Retention test

On the second day, the retention test was performed. Each rat was placed in the light compartment for 5 s, then the door was opened and the step-through latency during retention (STLr) and the time spent in the dark compartment (TDC) up to 300 s was measured. If the rat did not enter the dark compartment within 300 s, the retention test was terminated and a ceiling score of 300 s was recorded.

Electrophysiological recording and LTP induction

Following the two week recovery period and 10 days treatment in each experimental group, the rats were anesthetized with urethane (1.5 g/kg, intraperitoneally), and fixed in a stereotaxic apparatus for surgery, electrode inserting and field potential recording. During surgery, a heating pad was used to maintain the body temperature of the animal during the procedure. We drilled two small holes at the positions of stimulant and recording electrodes (0.008 in. in diameter, Teflon-coated stainless steel, A-M Systems, USA). The stimulating electrode was positioned in the perforant pathway (PP) [AP: -8.1 mm from bregma; ML: +4.3 mm from midline; DV: 3.2 mm from the skull surface]. The recording electrode was inserted in the dentate gyrus granular cell layer [AP:-3.8 mm from bregma; ML: +2.3 mm from midline; DV: 2.7-3.2 mm from the skull surface] according to the Paxinos and Watson atlas of the rat brain [40]. The electrodes were pulled down very gradually from cortex to the hippocampus, with the purpose of minimizing trauma to the brain tissue [42].

Input-output current profiles were obtained by stimulating the PP to determine the stimulus intensity to be used in each animal (40% maximal population spike (PS)). Single 0.1 ms biphasic square wave pulses were delivered through

constant current isolation units (A365, WPI Instruments, Waltham, MA) at a frequency of 0.1 Hz. The field potential recording was obtained in the granular cells of the dentate gyrus following stimulation of the PP [43].

An input/output (I/O) response curve was constructed using the intensity of single-pulse stimulation of the PP and averaging 10 responses per intensity in the DG. The evoked field potential response was equal to 50% of the maximum response for all subsequent stimuli. This baseline response recorded was considered as the time point "0 min" in measurements of the PS amplitude and field excitatory postsynaptic potential (fEPSP) slope just before high frequency stimulation (HFS). The HFS (400 Hz, 10 bursts of 20 stimuli, 0.2 ms stimulus duration, and 10 s inter-burst interval) induced LTP. After high-frequency stimulation, evoked responses were recorded at 5, 30, and 60 min and both fEPSP and PS were measured. PS amplitude was measured as the distance from the negative peak to halfway between two positive peaks. The fEPSP slope was measured as the slope of the rising part of the first positive peak.

Western blotting for Bax and Bcl-2

Western blot analysis was performed on the hippocampal tissues to determine the expression of Bax and Bcl-2. Immediately after LTP recording, the rats were sacrificed and the whole brain was quickly removed from the skull. Then, hippocampal tissues were dissected out, snap-frozen in liquid N2 immediately and finally stored at -80 °C until the time of tissue processing. The right frozen hippocampi were homogenized using 200 µL of lysis buffer [RIPA buffer and a protease inhibitor cocktail (1:20)] for an hour, and subsequently centrifuged at 12,000 g at 4 °C for 20 min. Protein concentration was measured with the BioRad protein assay. One hundred µg of the total protein from each sample was separated on a sodium dodecyl sulfate polyacrylamide gel and transferred to a nitrocellulose membrane. Next, the membrane was blocked with 5% non-fat dry milk (NFDM) and 0.1% Tween-20 in TBST buffer for an hour at room temperature and then incubated anti-Bcl-2 and anti-Bax (both 1:1000) antibodies overnight at 4 °C, and horseradish peroxidase (HRP)-conjugated secondary antibody (1:10000) for 1 h. The band detection was performed using an ECL Western Blotting Substrate Kit as a chemiluminescent substrate. Lastly, the density of the band was measured using Image-J software. β-actin antibody (1:1000) was used as a normalization control [32,33].

Statistical analysis

The data were expressed as mean±S.E.M and analyzed by SPSS 16.0 statistical software. Electrophysiology data including the amplitude of the PS and the slope of the EPSP were analyzed with one- and two-way analysis of variance (ANOVA). A Tukey post-hoc test was used where pairwise comparisons were performed. Furthermore, the data of behavioral and biochemical assessments were statistically analyzed using a one-way ANOVA followed by Tukey's post hoc tests for comparisons between groups. Statistical significance was set at $p<0.05$.

Results and Discussion

The analysis of the results showed that there was no significant difference in the STLa among the experimental groups in the first acquisition trial (before receiving the electrical shock). It was also observed that there were no significant differences in the number of trials during the first acquisition between the different experimental groups.

One-way ANOVA indicated that there were significant differences in the STLr between all groups in the retention test ($F (5, 48) = 43.25$, $P < 0.001$) (Figure 2a). Tukey test showed that the AD group had lower STLr than the control and sham groups ($p<0.001$). The STLr was significantly lower in AD group than that of the AD group receiving vitamin E ($p<0.001$), the AD group receiving *G. biloba* ($p<0.001$) and the AD group receiving *G. biloba* +vitamin E ($p<0.001$). In addition, animals in AD+vitamin E, AD+Gb and AD+Gb+vitamin E had smaller latency differences than animals in control and sham groups ($p<0.001$, $p<0.01$ and $p<0.001$ respectively).

In addition, a statistically significant difference in the TDC was detected among the experimental groups, by one-way ANOVA ($F (5, 48) = 20.33$, $p<0.001$) (Figure 2b). TDC in the AD group was significantly greater than that of control and sham groups ($p<0.001$). According to the results, TDC of the AD group was significantly greater than that of AD+vitamin E ($p<0.001$) and AD+Gb+vitamin E ($p<0.01$) groups.

Field potential recordings were obtained from the granular cell layer of the dentate gyrus following the high-frequency stimulation of the perforant

path. Figure 3 indicates sample traces from each group before and after the HFS. The traces of PS amplitude and fEPSP slope in the control, sham, AD+Vitamin E, AD+Gb and AD+vitamin E+Gb groups, increased following high-frequency stimulation, but not in the AD group.

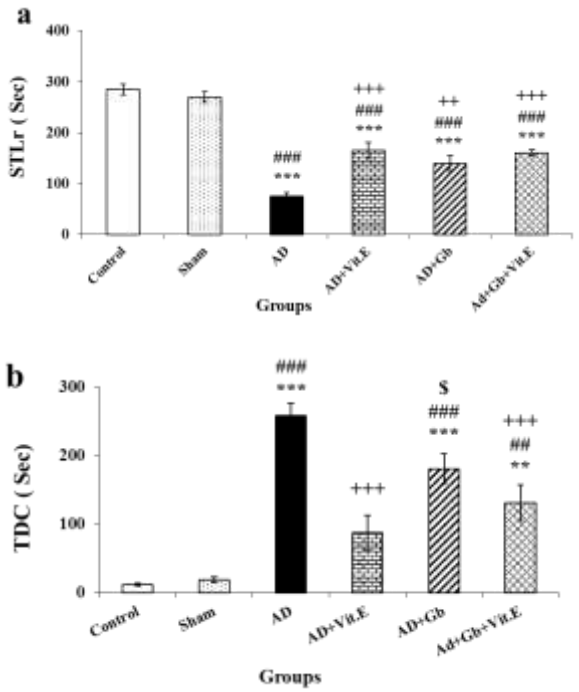

Figure 2. Effect of vitamin E, Gb (*Ginkgo biloba*) administration and co-administration of vitamin E and Gb treatment on the step through latency in retention (STLr) (a), and time spent in the dark compartment (TDC) (b) on the passive avoidance learning task**.** Data are shown as mean ± SEM; ** p<0.01; ***p<0.001 as compared with the control group. ## p<0.01; ### p<0.001 as compared with the sham group. ++p<0.01; +++p<0.001 as compared with the AD group; $ compared with the AD+Vitamin E

Figure 4 shows the effect of Aβ and treatments on the amplitude of the PS in the dentate gyrus. Statistical analysis with two-way ANOVA determined that there were significant differences in the PS amplitude among groups and time points (5, 30, and 60 min) points. Furthermore, as shown by Tukey test, the PS amplitude of the AD group was significantly lower than that of the control and sham groups at 5 min (p<0.05, p<0.01 respectively), at 30 and 60 min (p<0.05). There was no significant difference in PS amplitude between AD and vitamin E group. There was a significant reduction in the PS amplitude between AD compared to AD+Gb, AD+Gb+vitamin E groups at 5 min (p<0.05). There was no significant difference in PS amplitude between AD compare to AD+Gb, AD+Gb+vitamin E groups at 30 and 60 min.

Figure 5 illustrates the effects of Aβ and treatments on the fEPSP slope in the dentate gyrus granular cell synapses. In accordance to the amplitude of PS, there was a significant effect of treatment and time points in the slope of EPSP of the granular cell of dentate gyrus. There was no significant effect on the interaction between treatment time points. Tukey test showed that the fEPSP slope in the AD group significantly decreased compared to the control and sham groups at 5 min (p<0.05, p<0.05 respectively). The AD group has low fEPSP slope compared to control and sham groups at 30 min (p<0.05, p<0.05 respectively). The fEPSP slope of the Gb-treated AD group was significantly more than that of the AD group at 5 min (p<0.01). Additionally, there was no significant difference between AD compared to AD+vitamin E and AD+Gb+ vitamin E groups.

To investigate the protective effect of vitamin E and *Gingko biloba* on the Bcl2/Bax ratio in the AD rat, the Bcl-2 and Bax protein expression levels were detected by western blot analysis. Bax is one of the proteins that play an important role in developing apoptosis and Bcl2, as an antiapoptotic protein, has a vital role in controlling apoptosis pathway. The Bcl2/Bax ratio gives an index to the cell death or survival, after an apoptotic stimulus. Our results showed that Aβ injection significantly decreased Bcl-2 expression (p<0.0001) and increased Bax expression (p<0.0001). Therefore, the Bcl2/Bax ratio significantly decreased in the AD group compared to the control and sham groups (p<0.0001). In AD groups that received Gb, Vitamin E and Gb+vitamin E, this ratio increased (p< 0.0001) in comparison with the AD group and did not show a significant difference compared to the control group (Figure 6). The main findings of this study are as follows: (1) Intrahippocampal Aβ injection caused a decrease in the STLr and an increase in the TDC in the PAL test, and hence impaired memory. (2) Treatment with *G. biloba*, vitamin E and *G. biloba* +vitamin E improved the passive avoidance memory impairment caused by the Aβ injections. (3) Intrahippocampal Aβ injection reduced both components of LTP (fEPSP and PS) in the hippocampal dentate gyrus granule cells. (4) Treatment with *G. biloba*, vitamin E and *G. biloba* +vitamin E increased PS in Aβ-treated rats; (5) Intrahippocampal Aβ injection decreased Bcl-2 expression and increased Bax expression in the hippocampus.

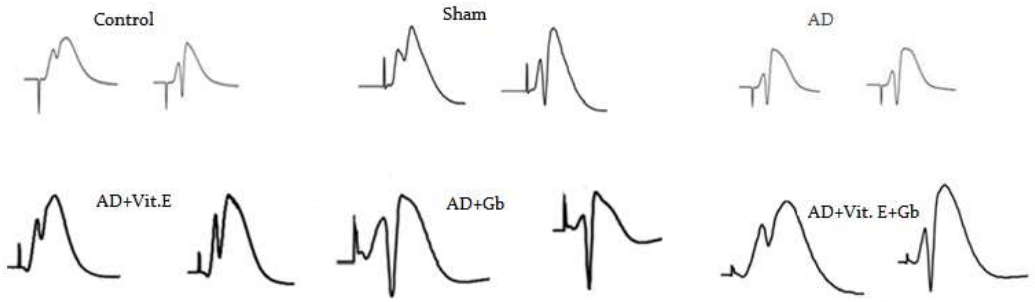

Figure 3. Sample traces of population spike (PS) amplitude and field excitatory postsynaptic potential (fEPSP) slope recorded in the perforant pathway to dentate gyrus before and after HFS (high-frequency stimulation).

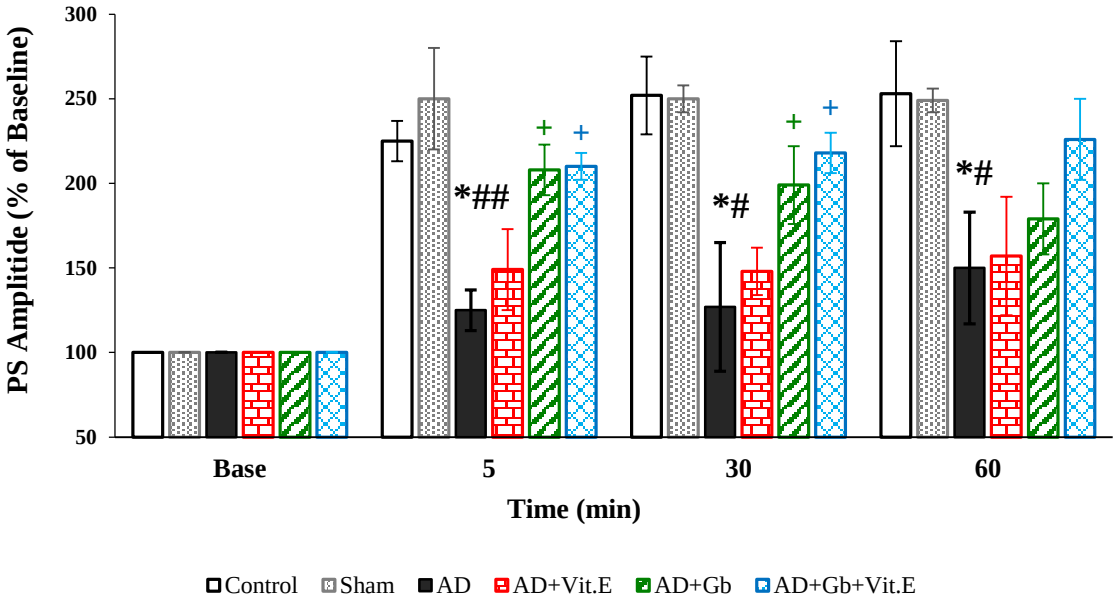

Figure 4. Time-dependent changes in the population spikes (PS) amplitude of dentate gyrus responses to perforant pathway stimulation following treatment of vitamin E, *Gingko biloba* and co-administration of vitamin E and *Gingko biloba*. Data are expressed as mean ± S.E.M. % of baseline. LTP of the PS amplitude in area DG granular cell synapses of the hippocampus are significantly different between groups except between AD and vitamin groups. *: $p<0.05$ significant difference (AD in compare with control) and, #: $p<0.05$, ##: $p<0.01$ significant difference (AD in compare with sham). +: $p<0.05$, significant difference (AD in compare with treatment).

(6) Treatment with *G. biloba*, vitamin E and *G. biloba*+vitamin E increased Bcl-2 expression and increased Bax expression in the hippocampus. Local injection of Aβ induce AD model in experimental animals [8,38]. Amyloid beta plays an important role in the pathophysiology of AD. In the present study, Aβ injection caused memory deficits, as demonstrated by performance on the PAL test. Our results is similar to others reported that injection of Aβ induced learning and memory impairment in rats [13]. Aβ injection impaired LTP in the granular cells of the dentate gyrus in the hippocampus. As mentioned previously, LTP is an increase in synaptic response following high-frequency electrical stimulation in the hippocampus and cortex [44]. Beta-amyloid impaired in vivo hippocampal LTP [8], and impaired LTP in the granular cells of the dentate gyrus [38]. These results suggest that memory deficits may be caused by synaptic dysfunction in AD.

We observed that intrahippocampal injection of Aβ decreased Bcl-2 expression and increased Bax expression in the hippocampus. Bcl-2 and Bax proteins regulate programmed cell death in nervous systems [15,45]. In agreement with our

results, the relative expression of Bax/Bcl-2 was significantly increased in Aβ1-40 (10 µg/µL) and Aβ1-42-injected rats [46,47].

The present study demonstrated that treatment with *G. biloba* improved Aβ- induced memory deficits, as measured by the PAL test. Consistent with our results, the *G. biloba* extract attenuated the amnesic effects of various substances in mice [48] and rats [31,49,50], and improved the cognitive impairment caused by β-amyloid in the rat model of AD [51].*Ginkgo biloba* can increase neurotransmitter release, particularly glutamate in the hippocampal nerve terminals and improve cognitive functions [52,53]. Additionally, our results indicated that the improved memory might be due to improved synaptic function and neuronal plasticity. Current data showed that treatment with *G. biloba* increased both fEPSP and PS in Aβ-treated rats. Treatment with *G. biloba* enhanced the synaptic plasticity in hippocampus of vascular dementia rats [54], aged rat [55], and in slices from aged mice [56].

In this study, treatment with *G. biloba* increased Bcl-2 and decreased Bax proteins in the hippocampus. The Bcl-2 protein family regulate neuronal apoptosis. Bax/Bcl-2 ratio is an important apoptotic index in neurons. A high Bax/Bcl-2 ratio is associated in AD [19]. Our finding is associated to previous studies that *G. biloba* (EGb761) increased Bcl-2 and decreased Bax protein levels in hippocampus tissue in rats [57] and both of young and aged mice [58,59]. The downregulation of Bax and upregulation of Bcl-2 moderate apoptosis induced by Aβ in cells [60,61].

The other finding from the current study showed that treatment with vitamin E improved the passive avoidance memory impairment caused by the Aβ injections. Previous studies reported the beneficial effect of vitamin E against streptozotocin [62], scopolamine [63] and propylthiouracil [64] in memory of animal. Current data showed that treatment with vitamins E increased PS amplitudes. Vitamin E alleviated LTP impairments by acting as an antioxidant, or by increasing dopamine and acetylcholine levels [34,60].

Another finding of the present study is that combination of vitamin E and *G. biloba* significantly attenuated AB-induced memory impairment, LTP , and decreased the Bax/Bcl-2 ratio in the hippocampus brain region studied, suggesting that antioxidants (from *G. biloba*) and free radical scavengers (from vitamin E) may prevent the neuronal apoptosis, increased neuronal plasticity and improve learning and memory.

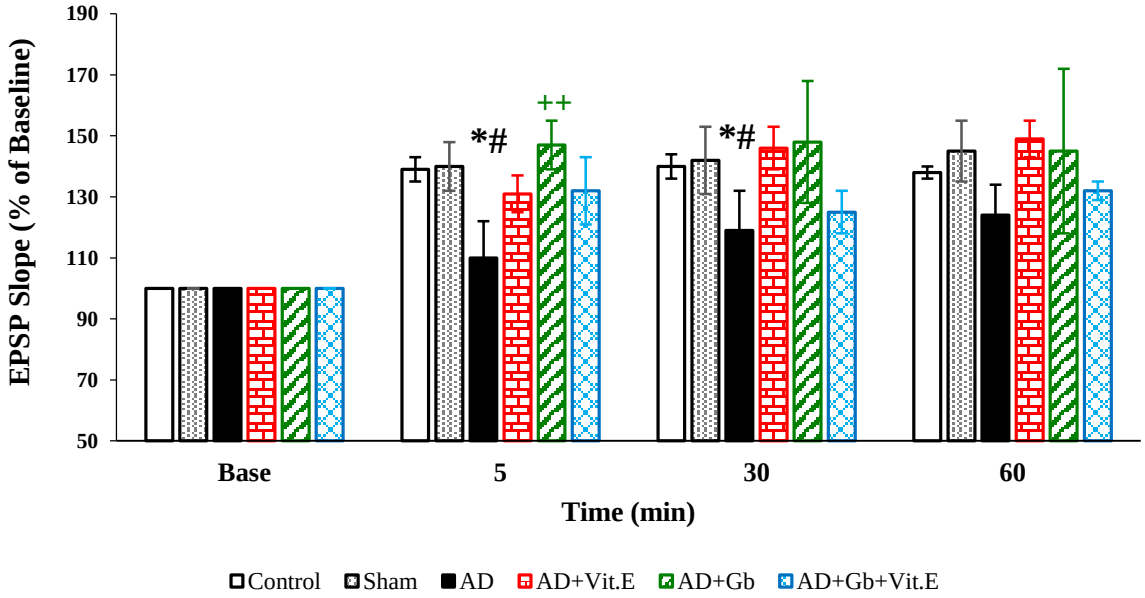

Figure 5. Time-dependent changes in the excitatory post-synaptic potentials (fEPSP) slope of dentate gyrus responses to perforant pathway stimulation following treatment of vitamin E, *Gingko biloba* and co-administration of vitamin E and *Gingko biloba*. Data are expressed as mean ± S.E.M. % of baseline. LTP of the EPSP slope in area DG granular cell synapses of the hippocampus was significantly different between groups except between AD+Gb+vitamin E and AD groups. *: $p<0.05$ significant difference (AD in compare with control). #: $p<0.05$ significant difference (AD in compare with sham). ++: $p<0.01$, significant difference (AD in compare with treatment).

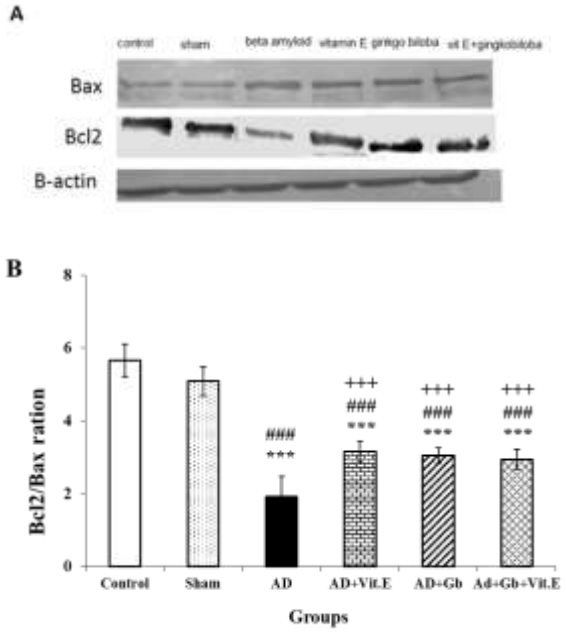

Figure 6. Western blot analysis for evaluating the effect of intrahippocampal injection of Aβ and Aβ plus vitamin E, Gb and Gb+vitamin E on Bax, Bcl2, and Bcl2/Bax ratio as apoptosis markers. Data are expressed as mean ± S.E.M.. A, The density of Bax and Bcl-2 bands for all groups. B. Bcl2/Bax ratio for all groups. ***p< 0.001 as compared with the control group. ### p< 0.001 as compared with the sham group. +++p< 0.001 as compared with the AD group.

Single treatment with vitamin E or *G. biloba* improved memory, hippocampal LTP and expression of Bax and Bcl-2 proteins. Their combination increased Bax/Bcl-2 ratio and PS amlitude of LTP. The Bax/Bcl-2 ratio change in degenerative diseases [16]. Aβ peptide increases Bax/bcl-2 ratio and induced apoptosis [18,19]. The co-treatment with vitamin E and *G. biloba* reversed the increase Bax/Bcl-2 ratio and attenuated apoptotic effect of Aβ peptide [65]. In the present study, the treatment period was ten days. However, a four- day treatment with *G. biloba* decreased Bax/bcl-2 ratio in all brain regions [58]. Long-term administration of *Ginkgo biloba* extract increased levels of monoamine in the hippocampus positively correlated to the retention of memory [53]. Significant results will be obtained after four weeks and two weeks time is not enough for the results to come up.

[65]. It seems that chronic treatment may cause suitable effects in learning and memory. The next studies are necessary for better understanding the effect of combined *G. biloba* and vitamin *E* therapy in AD.

Conclusion

In conclusion, *G. biloba* and vitamin E reversed the increase in Bax/ Bcl-2 ratio, improved memory deficiency and LTP impairment by Aβ. Combined treatment of vitamin E and *Ginkgo biloba* increased Bax/Bcl-2 ratio and PS amlitude of LTP. Further studies such as histological and molecular experiments with different time of treatment should be conducted to determine the mechanistic pathways of *G. biloba* and vitamin E in AD.

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Author contributions

Study concept and design: Siamak Shahidi and Sara Soleimani Asl; acquisition of data: Fatemeh Ghahremanitamadon; analysis and interpretation of data: Fatemeh Ghahremanitamadon, Simin Afshar, Nasrin Hashemi-Firouzi and Siamak Shahidi; administrative, technical and material supports: Siamak Shahidi, Sara soleimani Asl and Alireza Komaki; drafting of the manuscript: Fatemeh Ghahremanitamadon prepared the manuscript, Siamak Shahidi, Simin Afshar and Nasrin Hashemi-Firouzi were involved in critical revision of the manuscript for important intellectual content

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the accuracy and integrity of the paper content.

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Abbreviations

Aβ: beta-amyloid; ANOVA: analysis of variance; DG: dentate gyrus; fEPSP: field excitatory postsynaptic potential; IHP: intrahippocampal; LTP: long-term potentiation; PAL: passive avoidance learning; PP: perforant pathway; PS: Ppopulation spike; STLa: step-through latency during acquisition; STLr: step-through latency during retention; TDC: time spent in the dark compartment; SEM: standard error of the mean