Trigonelline Ameliorates Learning and Memory and Synaptic Plasticity Impairment in Intrahippocampal Amyloid Beta (1-40) Rat Model of Alzheimer's Disease

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Received: 06 Dec. 2017; Accepted: 13 Aug. 2018

Abstract- Intrahippocampal amyloid β (Aβ) negatively affects synaptic plasticity with subsequent impairment of learning and memory. Trigonelline is an alkaloid commonly found in fenugreek seeds and coffee beans with neuroprotective property and a promising agent for management of neurodegenerative disorders like Alzheimer's disease (AD). In the present study, the possible beneficial effect of trigonelline on the improvement of learning and memory and synaptic plasticity was evaluated in Aβ (1-40) rat model of AD. For modeling AD, aggregated Aβ (1-40) (10 μg/2 μl for each side) was bilaterally microinjected into the hippocampal CA1 area. Trigonelline was administered *p.o.* at a dose of 100 mg/kg. The results showed that trigonelline pretreatment of Aβ-microinjected rats ameliorates learning and memory deficit in passive avoidance task and spatial memory impairment in Morris water maze (MWM) paradigm. It also improved population spike (PS) amplitude and field excitatory post-synaptic potential (fEPSP) slope following application of high frequency stimulation (HFS) to induce long-term potentiation (LTP) in medial perforant-dentate gyrus pathway as an index of synaptic plasticity. Additionally, trigonelline mitigated hippocampal activity of acetylcholinesterase (AChE). In summary, trigonelline pretreatment of intrahippocampal Aβ-microinjected rats could ameliorate learning and memory impairment, partly through restoring hippocampal synaptic plasticity and AChE and it may be suggested as an adjunct and promising oral bioactive therapeutic agent that may prevent memory deterioration in AD.

Acta Med Iran 2018;56(10):625-634.

Keywords: Trigonelline; Alzheimer's disease; Amyloid β; Learning and memory; Synaptic plasticity; Long-term potentiation

Introduction

Alzheimer's disease (AD) is the most common form of dementia that affects millions of people worldwide and has become a major medical and social problem for developed and developing countries (1,2). AD is clinically characterized by progressive decline of cognitive function, in particular, the memory domain, finally leading to complete dependency and destruction. The key histopathological hallmarks of AD are extracellular neuritic plaques composed of β amyloid peptide (Aβ) and intracellular neurofibrillary tangles consisting of an abnormally phosphorylated form of the tau protein (3). Passive avoidance learning and memory deficits (4-6) and impairment of hippocampus-dependent spatial learning and memory have been reported in animal models of AD (7-9). Furthermore, alteration of hippocampal synaptic plasticity has already been described in AD (10). In this regard, Aβ as a neurotoxic mediator inhibits long-term potentiation (LTP) which is known as the functional unit of learning and memory processes (11,12). Cognitive impairment and memory decline in patients with early-stage AD occur before the development of prominent neuronal loss which may be associated with Aβ-induced synaptic dysfunctions such as alterations in LTP (13).

Trigonelline, a vitamin B3 precursor, is an alkaloid belonging to a group of pyridine betaines, commonly

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found in *Trigonella foenum-graecum* L. (fenugreek) seeds and coffee beans (14-16). To date, anti-diabetic properties of trigonelline and its beneficial influence on lipid profile have been proven (17,18). Also, this alkaloid has suggested as a potential neuroprotective agent, especially in AD (19) and as a constituent of coffee beans may be capable of improving regeneration of neurites in addition to memory improvement (15). Since most prescribed anti-AD drugs are cholinesterase inhibitors that act by increasing acetylcholine level in the brain (20), trigonelline has shown a weak AChE inhibitory activity (21). Recently, neuroprotective and anti-apoptotic effect of trigonelline in 6-hydroxydopamine-induced model of Parkinson's disease in the rat has been reported (22). Additionally, anti-inflammatory and anti-apoptotic effect of trigonelline in diabetic mice has also been reported (23). Overall, little work has been done on the effect of trigonelline in the brain in different models of human disorders. Therefore, we hypothesized whether oral administration of trigonelline could improve learning and memory deficits and LTP induction and maintenance in the dentate gyrus (DG).

Materials and Methods

Animals

Male albino Wistar rats (procured from laboratory animals breeding center of Iran University of Medical Sciences, Tehran, Iran) weighing 250-290 g were housed in Plexiglas cages with woodchip bedding in groups of 2-3 per cage at controlled room temperature (23±2º C) and a humidity of 30-40%) under standard 12-12 h light-dark cycle (the light period started at 07:00 a.m.). Food and water were available *ad libitum*. Procedures involving animals and their care were approved by Ethics Committee of Research Council of Iran University of Medical Sciences and conducted in compliance with the National Institutes of Health guidelines for the care and use of laboratory animals.

Aβ (1-40) preparation

Aβ (1-40) (Sigma-Aldrich, USA) was prepared as a stock solution in sterile 0.1 M phosphate-buffered saline (pH 7.4), and aliquots were stored at -20° C. Aβ solution was aggregated by incubation at 37° C for 4 days before use, as previously reported (24).

Stereotaxic surgery

On the day of surgery, the rats (n=10-12 per each group) were anesthetized with an intraperitoneal injection of mixed ketamine (100 mg/kg) and xylazine (10 mg/kg). The rats were mounted in a stereotaxic frame and after drilling two burr holes at coordinates: 3.5 mm posterior to the bregma, ±2 mm lateral to the sagittal suture, and 2.8 mm below the dura according to rat stereotaxic brain atlas (25), Aβ (1-40) solution (10 μg/2 μl) was bilaterally microinjected into the CA1 area of dorsal hippocampus. Sham operated rats received vehicle solution. Finally, the skin was sutured and the animals were monitored to recover in a warm box before returning to their home cages.

Experimental procedure

The rats (n=46) were randomly allocated to the following four groups: Sham, Trigonelline-pretreated Sham, Aβ, and Trigonelline-pretreated Aβ. Trigonelline (Sigma-Aldrich, USA) was dissolved in distilled water and administered *p.o.* at a dose of 100 mg/kg, starting 3 days before the surgery till 1 h pre-surgery. The dose of trigonelline was chosen from our earlier study on its neuroprotective and anti-apoptotic effect in 6-hydroxydopamine rat model of Parkinson's disease (22). Sham animals received an equivalent volume of the vehicles. Experimental scheme of the study has been depicted in figure 1.

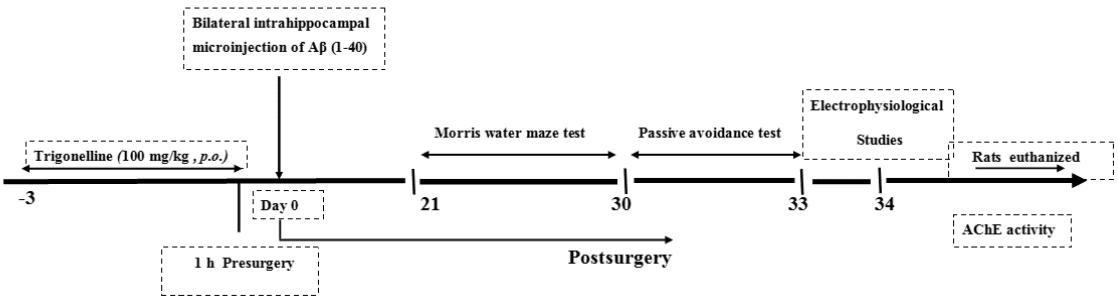

Figure 1. Experimental design of the study. Trigonelline (100 mg/kg/day) was administered to rats with bilateral intrahippocampal Aβ (1-40) (10 μg/2 μl), started 3 days before the surgery till 1 h pre-surgery

Passive avoidance test

This test was conducted according to previous reports (26-28). The apparatus consisted of an illuminated chamber connected to a dark chamber by a guillotine door. Electric shocks were delivered to the floor grid by an isolated stimulator. In the first and second days of testing, each rat was placed into the apparatus and left for 10 min. to habituate to the apparatus. On the third day, an acquisition trial was performed. Rats were individually placed in the illuminated chamber. After a habituation period (10 min), the guillotine door was opened, and after the rat entering the dark chamber, the door was closed, and an inescapable electric shock (1 mA, 1 s once) was delivered. In this trial, the initial latency (IL) of entrance into the dark chamber was recorded, and rats with initial latencies greater than 60 s were excluded from the study. Twenty-four hours later, each rat was placed in the illuminated chamber for retention trial. The interval between the placement in the illuminated chamber and the entry into the dark chamber was measured as a step-through latency (STL) (up to a maximum of 600 s as the cutoff time).

Morris water maze test

The Morris water maze (MWM) protocol has been described previously (9,29). Briefly, the maze consisted of a black cylindrical pool (a diameter of 140 cm and a height of 70 cm) with a uniform inner surface. The pool was filled with water at 21° C (±1° C) to a height of 30 cm. The maze was divided geographically into four equal quadrants and release points that were designed at each quadrant as N, E, S, and W. A hidden circular platform (15 cm×10 cm) was located in the center of the southwest quadrant, submerged 1.5 cm beneath the surface of the water. The platform was the same color as the pool wall, so it was invisible to rats. Fixed, extra-maze visual cues were present at various locations around the maze. An indirect illumination in the experimental room was provided by white neon tubes fixed on the walls. Each experiment comprised of one session with four trials lasting 60 s during five consecutive days. A 60 s probe test with a removed platform was given 24 h after the completion of the last session. For each trial, the rat was placed in the water facing the pool at one of four pseudo-randomly determined starting positions. Once the rat has found and mounted the escape platform, it was permitted to remain on the platform for 30 s. The rat was guided to the platform by the experimenter if it failed to find it within 60 s. A CCD camera was fixed above the center of the maze so that the animal motion could be recorded and sent to the computer. The path of animal's swimming was automatically recorded by a computerized system (EthoVision, Noldus, Version 7). The total traveled distance was the parameter analyzed in the acquisition phase and the number of frequency and time spent in target zone were analyzed for probe test. In all groups, one rat was deemed to be 'poor performer' and was excluded from the study.

Electrophysiological experiments

Medial perforant-dentate gyrus LTP was recorded under anesthesia with *i.p.* injection of 1.5 g/kg of urethane. The rats were placed in the stereotaxic frame for surgery, and rectal temperature was maintained at 37±0.5º C with an automatic heating pad. Teflon-coated recording and bipolar stimulating electrodes (stainless steel wire, a bare diameter of 0.125 mm, Advent, UK) positioned stereotaxically so as to selectively stimulate the medial perforant path while recording the dentate gyrus. The electrode stimulating the medial perforant path was implanted 4.2 mm lateral to the true lambda. A recording electrode was implanted ipsilaterally 3.8 mm posterior and 2.2 mm lateral to the bregma. The electrical signals from the DG were amplified 1000-fold, digitized at 10 kHz, and band-pass filtered at 0.1 Hz-10 kHz. The field potential recordings were started at least 20 min after placing the stimulation and recording electrodes. All the stimuli were biphasic square wave pulses (200 ms width), and their intensities for baseline recording were set at the current that evoked 40% of the maximum population spike amplitude (PSA). Test stimuli (0.1 Hz) were delivered at 10 s intervals to monitor field excitatory postsynaptic potentials (fEPSP) and population spike (PS). The strength of a field potential was evaluated from the slope of the EPSP and amplitude of the PS. The PS amplitude was measured by averaging the distance from the negative peak to the preceding peak and the following positive peak. The maximal EPSP slope was obtained on the first positive deflection of the field potential. After stable baseline recording for at least 60 min, the LTP was induced by delivery of high-frequency stimulation (HFS) (HFS: 0.5 ms stimulus duration, 10 trains of 10 pulses at 200 Hz and the trains were delivered once every 10 s) and after the tetanic stimuli, the baseline stimulation was resumed and recording continued for more than 1 h.

Determination of hippocampal AChE activity

Anesthetized rats were decapitated in a guillotine, and their brains were immediately removed. The hippocampus contralateral to the recording side was

punched out, and tissue homogenate was prepared in ice-cold lysis buffer in the presence of protease inhibitor cocktail (Sigma-Aldrich, USA) to make a 5% lysate. Each homogenate was centrifuged (1000 *g*, 4º C, 10 min), and an aliquot of the supernatant was stored at -80° C until used. The activity of AChE was assessed according to the method recommended by Ellman (30). In this regard, 20 µl of aliquoted homogenate was added to 3 ml of 0.01 M sodium phosphate buffer (pH 8), 0.10 ml of acetylthiocholine iodide and 0.10 ml of DTNB (Ellman reagent) and the change in absorbance was measured at 412 nm using a spectrophotometer. Changes in absorbance were recorded for 10 min at 2-min intervals, and enzyme activity was reported as mM of substrate hydrolyzed/min/g protein.

The protein concentration of the supernatant was measured by the Bradford method using bovine serum albumin as the standard (31).

Data analysis

For Morris water maze and electrophysiological comparisons, data were analyzed using repeated measures two-way ANOVA, with Tukey's *post-hoc* test to discriminate between groups. Passive avoidance test and AChE reactivity test data were analyzed by one-way ANOVA followed by Tukey's *post-hoc* test. All results have been shown as means±SEM. In all statistical comparisons, $P<0.05$ was statistically considered significant.

Results

Passive avoidance test

Figure 2 shows the performance of rats in the passive avoidance test as determined by IL and STL. Regarding IL, no significant differences were found out amongst the groups. Regarding STL, there was a significant difference between the groups (F (3,39)=4.663; P=0.0109). In this respect, Aβ group developed a significant impairment in retention and recall in passive avoidance test relative to Sham ($P<0.05$), as it was evident by a lower STL and trigonelline pretreatment of Aβ group at a dose of 100 mg/kg significantly improved STL relative to vehicle-pretreated Aβ group ($P<0.05$).

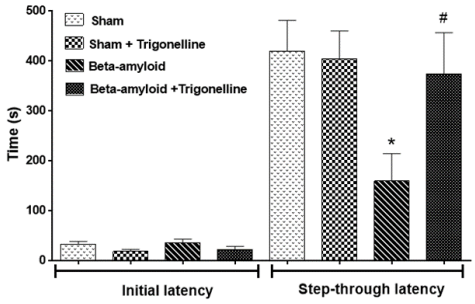

Figure 2. Initial (IL) and step-through (STL) latencies in passive avoidance test. Values are expressed as mean ± S.E.M. * $P<0.05$ (vs. Sham); # $P < 0.05$ (vs. Aβ)

Morris water maze (MWM)

Distances traveled to find the hidden platform during the acquisition phase of the experiment is presented in Figure 3. The repeated-measures ANOVA showed that the rats in groups of sham, sham+trigonelline and Aβ+trigonelline progressively improved their ability to locate the platform over the 5 days of training, indicating that they learned the spatial navigation task [F (5,20)=6.087; P=0.0014 for sham group; F (5,20)=3.102; P=0.031 for sham+trigonelline; F (5,20)=2.762; P=0.047 for Aβ+trigonelline]. Meanwhile, rats in Aβ group did not improve their ability to locate the platform over the 5 days of training which is possibly due to the impairment induced by Aβ [F (5,20)=1. 619; P=0. 20]. When the two-way ANOVA was applied to values for distances traveled to find the hidden platform during the learning phase, it revealed a statistically significant influence of the factor days (F (4,20)=8.577, P=0.0003) and interaction between treatment×days (F (12,60)=2.753, P=0.0048), while the factor treatment was not significant (F (3,15)=0. 4275, P=0.73). After analyzing the interaction with one way ANOVA followed by p*ost hoc* Tukey's test, it was revealed that Aβ group had a significantly longer distance to find the platform than sham group on days 1 and 2 (F (6,60)=0.4358; $P<0.05$ and F (6,60)=0.1528; $P<0.05$ on days 1 and 2, respectively, which indicates the deleterious effects of Aβ on learning process. Treatment with trigonelline reversed Aβ-induced impairment, only on day 5 (F (6,60)=3.995; $P<0.05$).

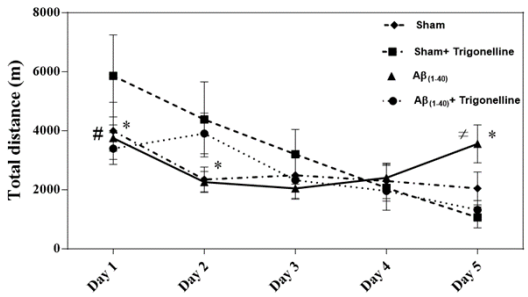

Figure 3. Performance of rats in Sham, Sham+Trigonelline, Aβ, and Aβ+Trigonelline) groups in the Morris water maze task. The figure shows distance traveled to find the hidden platform of four consecutive trials throughout the training period. Values are presented as means±S.E.M. * *P*<0.05 (versus sham), # *P*<0.05 (versus Aβ)

The results of the probe-trial tests are presented in figure 4. The one-way ANOVA applied to the data obtained from the probe test revealed a significant effect of treatment on the latency to first entry to the target region (F (3,23)=3.622; *P*=0.0282). Analysis of time in the target quadrant revealed that there were significant differences between sham and Aβ groups (F (3,22)=4.267, *P*=0.0161). Moreover, there was a significant difference between Aβ+trigonelline and Aβ groups regarding the time in the target quadrant (Figure 4A).

The one-way ANOVA applied to the data obtained from the frequency in the target region revealed a significant effect between the groups (F (3,22)=4.267; *P*=0.0161). Results of treatment on frequency in the target region revealed that there was a significant difference between Aβ+trigonellin and Aβ groups (*P*<0.05) (Figure 4B).

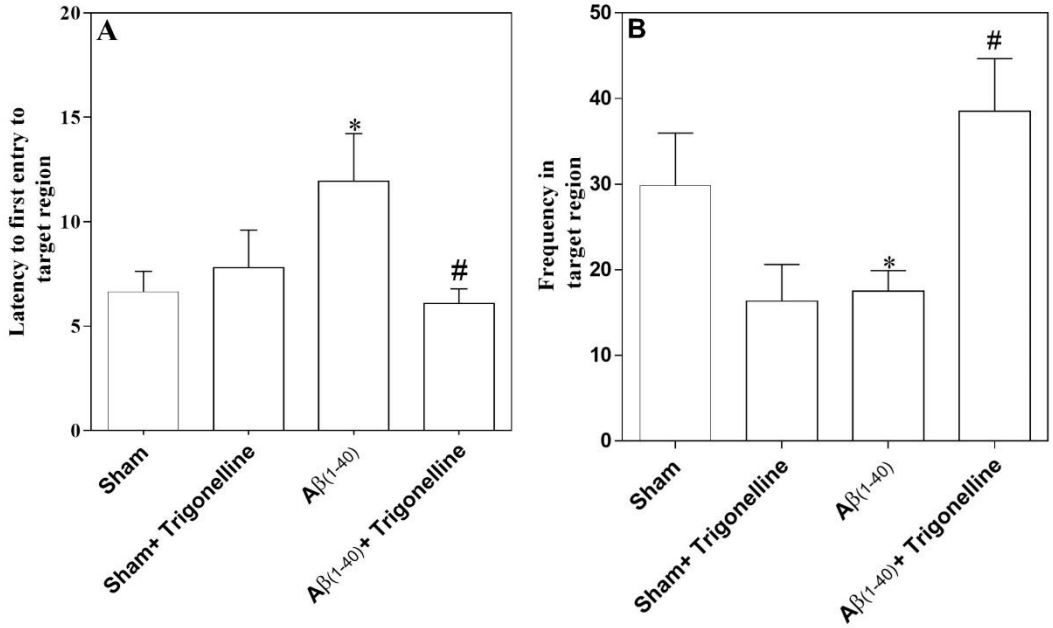

Figure 4. Memory retention as measured by the MWM task using the probe trial 1 day after the last acquisition test. (A) The mean latency to reach the previous location of the platform of the 60 s probe-trial. (B) The frequency in the target region (number of crossings) on the previous position of a platform had previously visited. * *P*<0.05 (versus sham), # *P*<0.05 (versus Aβ).Values are means±S.E.M

Electrophysiological findings

As illustrated in figure 5, the effect of trigonelline treatment on LTP induction and maintenance in the dentate gyrus was determined. A repeated measure ANOVA followed by *post hoc* test revealed that the PS-LTP after tetanization (HFS) was significantly lower in the Aβ group relative to the sham group (*P*<0.01). However, there was no significant difference between the groups sham and sham+trigonelline in this respect. The EPSP-LTP after tetanization was significantly lower in the Aβ group with respect to the sham group (*P*<0.01). High-frequency stimulation (200 Hz) of medial perforant path produced a long-lasting synaptic potentiation in Aβ+trigonelline group as compared to Aβ one (*P*<0.001) up to 60 min after HFS (Figures 5A-E).

Hippocampal acetylcholinesterase (AChE) activity

The sham group receiving trigonelline did not show a

significant change of hippocampal AChE activity (67.3±9.8 mM/substrate hydrolyzed/min/g protein; *P*>0.05) as compared to the sham group (64.5±8.7 mM/substrate hydrolyzed/min/g protein). In contrast, rats in Aβ group showed a significantly higher level of AChE activity (143.5±12.7 mM/substrate hydrolyzed/min/g protein) as compared to the sham group and Aβ+trigonelline group (95.5±10.3 mM/substrate hydrolyzed/min/g protein) had a lower level of enzyme activity relative to Aβ group (*P*<0.05) (Figure 6).

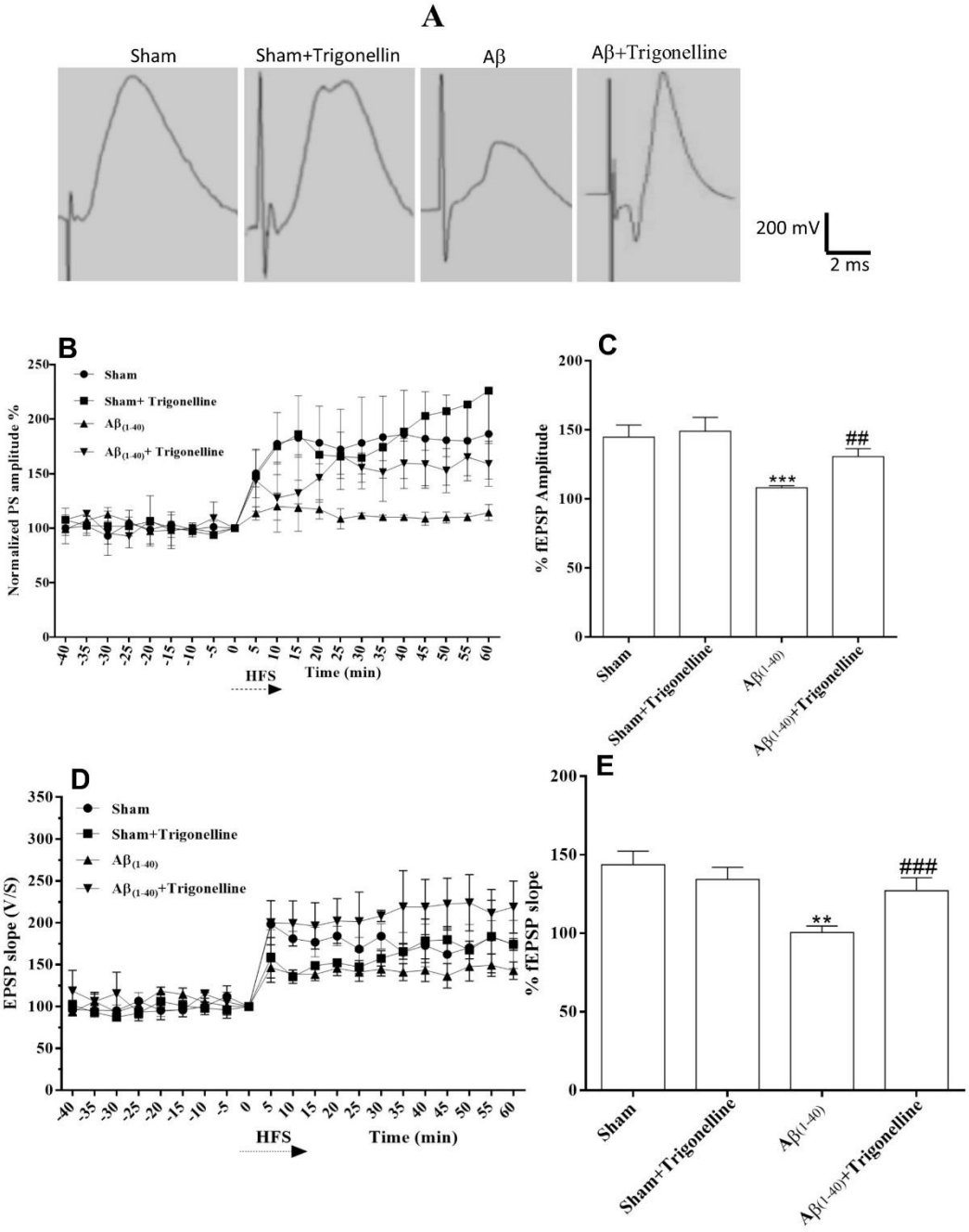

Figure 5. The normalized PS amplitude and EPSP slope in different groups before and after HFS. (A) Recorded traces from different groups. Recordings show changes in LTP recording for 60 min after HFS; each recording is the average of 10 consecutive recordings in 100 s with an interval of 10 s. (B) The normalized PS amplitude up to 60 min after HFS. (D) The fEPSP slope after tetanization up to 60 min after HFS. (C and E) Histograms showing the percentage of fEPSP amplitude or fEPSP slope in 60 min normalized to the baseline response after HFS in different groups. ** $P < 0.01$, *** $P < 0.005$ (versus sham); ## $P < 0.01$, ### $P < 0.005$ (versus Aβ).Values are means ± S.E.M

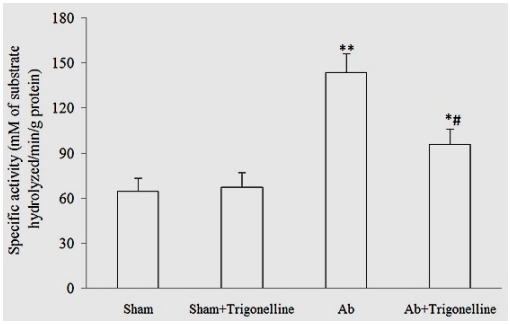

Figure 6. Acetylcholinesterase activity in hippocampal homogenate from different groups. * $P < 0.05$, ** $P < 0.01$ (versus sham); # $P < 0.05$ (versus Aβ).Values are means±S.E.M

Discussion

This work demonstrated that microinjection of Aβ (1-40) into the rat CA1 area of the hippocampus impairs synaptic plasticity and LTP as the molecular basis of learning and memory and trigonelline pretreatment at a dose of 100 mg/kg significantly ameliorates these abnormalities. Aβ plays an important role in the pathophysiology of AD and a close correlation exits between Aβ and the neurodegeneration process observed in AD (32). Nitta and colleagues have shown poor performance of Aβ-microinjected rats in Morris water maze task (5). The deposition of Aβ in the brain tissue is responsible for impairment of learning and memory and Aβ could induce a reliable animal model of AD (33). The key brain regions involved in navigation in the MWM task include the striatum, the frontal lobe, and the hippocampus (7,8). In the present study, the bilateral intrahippocampal microinjection of Aβ (1-40) induced a significant learning and memory disturbance in the passive avoidance and MWM tasks in the rat. Our results showed that administration of trigonelline to Aβ group improved learning and memory parameters in used tasks. Our results are consistent with other studies as Tohda and colleagues (15) reported trigonelline could affect the impairment of spatial memory induced by Aβ (25-35). They showed that the number of crossings over a previous platform position significantly decrease in the Aβ (25-35) microinjected group and recover by trigonelline treatment. Memory retention improvement due to medicinal plants constituents like trigonelline has also been reported before (4). Although the multifactorial pathogenesis of cognitive and memory impairments in AD has not yet completely been understood, but in recent years the most popular pathophysiological approaches have suggested that AD is characterized by low levels of the neurotransmitter acetylcholine (ACh), increased oxidative stress, high levels of some metal ions and overproduction and aggregation of Aβ (34-36), However, AD is also associated with neuronal loss in the hippocampus and this is functionally well-correlated with impaired learning and memory. These changes are preceded by significant increases in the hippocampal expression of some proteins such as neuron growth factor (NGF) and brain-derived neurotrophic factor (BDNF). Ivanov and colleagues showed that Aβ at a concentration 50 of nM completely abolishes LTP at the medial perforant -dentate gyrus pathway (37). Since trigonelline is a nicotinic acid derivative and nicotinic acid could elevate mRNA expression of NGF and BDNF, this could be considered as a possible mechanism involved in the neuroprotective effects of trigonelline (38-42). These reports are associated with our results about trigonelline. Our results also showed that trigonelline ameliorates LTP after HFS in Aβ+Trigonelline group and improves learning and memory in this group.

LTP is considered as a major synaptic mechanism for evaluating long-term synaptic plasticity in rodents. Post-tetanic LTP has been considered to be a physiological form of synaptic plasticity and its occurrence either in cortical or in subcortical areas has been regarded as a cellular substrate for learning and memory (43). In accordance to earlier studies, LTP induction and maintenance is significantly impaired in AD animals after tetanic stimulation (11,12) and in our study trigonelline treatment prevented the abnormal changes in hippocampal synaptic plasticity induced by Aβ. The stress-activated p38 MAPK plays important roles in transducing stress-related signals, including beta-amyloid toxicity, by phosphorylating intracellular enzymes, transcription factors, and cytosolic proteins involved in apoptosis, inflammatory cytokine production (44,45), and synaptic plasticity (46). There is accumulating evidence that p38 MAPK plays a role in AD pathophysiology (47-50) and suggest a role of p38 MAPK in synaptic dysfunction caused by oligomeric Aβ. The hypothesis has been raised that Aβ induces synaptic dysfunction in entorhinal cortex via activation of downstream p38

MAPK signaling (50). Zhue and Zhue in 2012 showed that phosphorylated p38 MAPK protein expression in sciatic nerve significantly increased in diabetic rats relative to non-diabetic control ones and chronic treatment with trigonelline significantly decreased the up-regulated phosphorylated p38 MAPK protein expression in diabetic sciatic nerve (19), which may have also happened in our research study.

AD is also associated with loss of cholinergic function, which affects memory, learning, and behavior. A large part of the strategies for treating AD has been based on the cholinergic hypothesis, which postulates that memory loss in Alzheimer's patients is associated with a deficit of cholinergic function in the brain (51). The loss of cholinergic neurons leads to the progressive reduction of ACh in the brain and the ensuing cognitive deficit in AD (52). An *in vitro* study using crude fenugreek extract showed its AChE inhibitory property (21). Oral ingestion of 10-100 mg/kg of a fenugreek seed extract (containing trigenolline as much as 82%) has failed to show anticholinergic effects in rats (53). However, researches have obtained contradictory results after administration of trigonelline. In this regard, *in vitro* AChE inhibitory activity of trigonelline was measured using Ellman's method in a 96-well microplate assay and a thin layer chromatography bioassay and it was shown that trigonelline could weakly inhibit AChE (21). In contrast, Orhan and colleagues found that trigonelline in an *in vitro* setting did not inhibit AChE or butyrylcholinesterase using Ellman method in an enzyme-linked immunosorbent assay (54). In the current study, our result that trigonelline can inhibit AChE activity and decrease the effectiveness of Aβ is possibly exerted due to its direct and indirect effects on the enzyme, and this has led to its improvement of learning and memory in Aβ-microinjected rats. However, more researches are warranted to determine the exact effect of this alkaloid on cholinesterase expression and activity.

In conclusion, trigonelline pretreatment of intrahippocampal Aβ-microinjected rats could ameliorate learning and memory impairment, partly through restoring hippocampal synaptic plasticity and AChE and it may be suggested as an adjunct and promising oral bioactive therapeutic agent that may prevent memory deterioration in AD.

Acknowledgments

This study was part of a Ph.D. thesis project that was approved and financially supported by Physiology Research Center affiliated to Iran University of Medical Sciences in 2014 (grant # 93-03-130-24998).

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