

Antiamnesic Effects of Walnuts Consumption on Scopolamine-Induced Memory Impairments in Rats

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ABSTRACT

Introduction: Alzheimer's disease (AD) is an age-related neurodegenerative disease, which impairs memory and cognitive function. Walnuts are a dietary source of polyphenols, antioxidants and other compounds with health beneficial effects. These characteristic of walnuts make them perfect candidates for evaluation of their possible effects on neurodegenerative diseases. Therefore the present study was designed to investigate the effects of walnuts consumption (2%, 6% and 9% walnut diets) on memory enhancement and acetylcholinesterase (AChE) activity of brain in scopolamine-induced amnesic rats.

Methods: Learning, memory and locomotor activity parameters were evaluated using Morris water maze (MWM), passive avoidance and rotarod tests.

Results: Our results showed that consumption of walnuts at doses of 6% and 9% significantly restored the scopolamine-induced memory impairments in the MWM and passive avoidance tests. Moreover, the potential of walnuts to prevent scopolamine neurotoxicity was also reflected by the decreased AChE activity in the whole brain in comparison with the scopolamine group.

Discussion: These results suggest that walnuts may be useful against memory impairment and it may exert these anti-amnesic activities via inhibition of AChE activity in the brain. It would be worthwhile to explore the potential of this nut and its active components in the management of the AD.

1. Introduction

Memory is the ability of an individual to record events and information and retain them over short and long periods of time (Dunning & During, 2003). Alzheimer's disease (AD) is known as the most common neurodegenerative disease. In this disease, progressive decline in cognitive function due to degeneration of the cholinergic neurons results in dementia (Cummings & Kaufer, 1996). Although there are several neuronal pathways and neurotransmitters which involve in formation of the memory, but it has been suggested that

acetylcholine (ACh) plays a more important role in shifting between encoding and retrieval of the memory (Klinkenberg & Blokland, 2010). Therefore, basic studies and also clinical drug trials in patients with AD have focused on drugs that increase levels of ACh in the brain to compensate for losses of cholinergic function of the brain. These drugs have included ACh precursors, muscarinic agonists, and acetylcholinesterase (AChE) inhibitors (Giacobini, 2000; Livingston & Katona, 2000). Since, these drugs may cause some undesired side effects, It is worthwhile to do further investigations to find AChE inhibitors with fewer side effects (Chattipakorn et al., 2007).

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Although the establishment of basic and clinical pharmacology as leading branches of medicine, has resulted in rapid decline of the herbal medicines, but they are still of interest in many diseases, in particular, psychiatric and neurological disorders (Akhondzadeh & Abbasi, 2006). Edible nuts are globally popular, and are valued for their sensory, nutritional, and health attributes (Venkatachalam & Sathe, 2006). The *Juglans* genus (family juglandaceae) consists of several species and is widely distributed throughout the world. The walnut tree (*Juglans regia* L) as the most well-known member can be found in temperate areas of the world (Pereira et al., 2007). In Iran, walnut trees are distributed in most places of the country. Nuts are very popular and largely consumed in Persian diet. Walnuts contain neuroprotective substances including vitamin E, folate and melatonin (Fukuda, Ito & Yoshida, 2003; Reiter, Manchester, & Tan, 2005). Moreover, traditional use of walnuts as a nootropic substance is well-established (Savage, 2001). Nowadays these nuts are receiving increasing interest due to their high content of antioxidants (Vinson & Cai, 2012). They are the first among all nuts (Pellegrini et al., 2006), and the second among all the dietary plants with antioxidant content (Halvorsen et al., 2002). On the other hand, a well-known connection exists between the antioxidant intake and a reduced incidence of dementia, AD and cognitive decline (Grundman & Delaney, 2002). These characteristics of walnuts make them perfect candidates for improving the memory loss.

Scopolamine is a muscarinic receptor antagonist which is traditionally used to evaluate the anti-amnesic activity of herbal agents on learning and memory of rodents (Beatty, Butters & Janowsky, 1986). It produces a reversible impairment in maintaining attention, information processing and also in the acquisition of new knowledge in both rodents and human subjects (Bejar, Wang & Weinstock, 1999; Wesnes et al., 1991).

Putting this all together, the present study was designed to investigate the beneficial effects of walnuts consumption on scopolamine-induced memory impairment in rats. Moreover, the brain AChE activity was also evaluated to determine the possible anticholinesterase effects of these nuts.

2. Methods

2.1. Animals

Male Wistar rats weighing 180-220 g were obtained from the colony maintained by Kerman Neuroscience Research Center animal house. Animals were ran-

domly distributed in different experimental groups and were caged individually under constant temperature (23 ± 1 °C), 12-h light-dark cycle (light on at 07:00). All experimental protocols and treatments were approved by the Ethical Committee of Kerman Neuroscience Research Center (EC/KNRC/91-24) according to the "NIH Guide for the Care and Use of Laboratory Animals".

2.2. Drugs

Scopolamine bromide was from Merck (Darmstadt, Germany). Acetylthiocholine iodide and 5,5'-dithio-bis-nitro-benzoic acid (DTNB) were obtained from Sigma (St. Louis, USA). Piracetam was supplied by Darou Pakhsh Pharmaceutical Company (Tehran, Iran). Piracetam and scopolamine were dissolved in 0.9% physiological saline for i.p. injection.

2.3. Morris water maze (MWM) test

The MWM test was performed according to the method of Frick et al., (Frick, Stillner, & Berger-Sweeney, 2000). The test was carried out in a black circular pool (160 cm in diameter and 80 cm in height) filled with water to a depth of 40 cm (21 ± 2 °C). The pool was divided into four equal quadrants. A platform (10 cm in diameter) was submerged 1.5 cm below the surface of the water in the center of one of the quadrants. The experiment was performed in a dimly lit room with some visual cues around the maze. Performance of each animal was monitored by a video tracing system (Ethovision, Noldus Information Technology, the Netherlands).

Twenty four hours prior to the start of the test, rats were habituated to the pool by allowing them to swim for 60 seconds in the absence of the platform. In this protocol, each rat accomplished three block sessions with inter-block intervals of 30 min. Each block per se, consisted of four successive trials with 60 seconds duration. On each trial, rats were randomly released into the water from one of the four quadrants facing the wall of the maze. During acquisition, the location of the platform remained constant and rats were allowed to swim to the hidden platform. When animal found the platform, it was permitted to remain there for 30 seconds and then returned to its cage for a 30 second inter-trial interval. If the rat failed to find the platform within 60 seconds, it was guided toward the platform. The time spent and distance moved from the starting point to the platform were collected and analyzed later. Two hours later, spatial memory was examined with a single probe trial. During this trial, the platform was removed and the rats were allowed to swim for a 60 second period. The time spent

and distance moved in the quadrant where the platform was previously located were analyzed as a measure of spatial memory retention.

Following the probe trial, a visible platform test was performed for each rat to assess whether any motivational factors interfered with the rats' ability to escape to a visible platform. The platform was raised two cm above the water surface and became visible with an aluminum foil. The animal's ability to escape to the visible platform was evaluated by a blind observer.

2.4. Passive avoidance test

An apparatus with two equal compartments (20cm×40cm×20 cm) separated by a guillotine door was used. During the habituation phase, rats were placed in the light compartment, facing away from the door. Five seconds later, the door was raised and the latency to enter the dark compartment was recorded for each animal. If animals did not enter the dark compartment in 120 seconds, they were excluded from the experiment. Two hours later, the training phase was performed and each animal was placed into the light compartment. After 5 seconds, the door was raised and the latency to enter the dark compartment was measured. Once the animal entered the dark side completely (all four paws), the door was closed immediately and a 0.5 mA unavoidable shock was delivered to the animal's feet for 2 seconds. The rat was then removed to the cage and the procedure was repeated 5 min later. Training was terminated when the rat remained in the light compartment for 5 consecutive minutes.

Twenty-four hours after the training, retention phase of the test was performed. Rats were placed in the light compartment and 5 seconds later, the door was raised. The latency for entering into the dark (shock) compartment was measured as step-through latency (STL). The experiment was continued for 300 s. Memory was measured as the tendency of animals for avoiding the dark compartment.

2.5. Rotarod test

The motor performances of rats were evaluated by an accelerating rotating rod (TSE systems, 3375-4R, Germany). Rats were placed on the elevated rod (6 cm in diameter) and the rod was accelerated from a minimum speed of 10 to 60 rpm. The cut-off time was set to 300 seconds and the latency to fall was measured for all animals. The procedure was repeated three times for each rat. The mean time was calculated for each rat and

was used as the value of the test. Rats were given 30 min inter-trial rest intervals to avoid fatigue. Animals were familiarized with the procedure three times prior to the start of the experiment (Shabani, Hosseinmardi, Haghani, Shaibani & Janahmadi, 2011).

2.6. Experimental design

Animals were categorized into six groups and separate animals were used for each behavioral experiment. Amnesia was induced by scopolamine injection (1 mg/kg, i.p.) 30 min before the behavioral experiments. All groups except the control group received scopolamine. Group I (control) received the vehicle only (0.9% physiological saline). Group II (scopolamine) received a single dose of scopolamine. Group III (piracetam) received piracetam (500 mg/kg, i.p.) as the positive control. Antiamnesic and memory-enhancing properties of piracetam have been shown in numerous studies. Piracetam and piracetam-like nootropics are capable of reversing scopolamine-induced amnesia (Gouliaviev & Senning, 1994; Lenegre, Chermat, Avril, Steru, & Porsolt, 1988). Groups IV–VI (walnut 2%, walnut 6% and walnut 9%) were pretreated orally for 4 weeks with fresh and daily supplied walnuts; while control, scopolamine and piracetam groups were fed by standard food (20 g daily). The walnuts were purchased from a local market and authenticated at the Biology Department of Shahid Bahonar University of Kerman, Kerman, Iran. The animals were fasted for 24 h prior to the first day of walnuts consumption. Walnuts were combined with the standard diet of animals (2%, 6% and 9% of daily food). All groups had free access to water. The drug doses were selected according to previous studies (Alikatte, Akondi, Yerragunta, Veerareddy, & Palle, 2012; Kumar, Singh, Muruganandam, & Bhattacharya, 2000). Body weight and food intake of all rats were monitored throughout the experimental period.

2.7. Biochemical assay

At the end of behavioral experiments, animals of each group were randomly divided into two subgroups. In first subgroups, sixty min after assessing the learning, memory, and locomotor activity paradigms, rats were euthanized by cervical decapitation. The whole brain was immediately removed and stored in -80°C until the AChE activity assay. Brains were homogenized by an ultrasonic homogenizer (Hielscher, UP200H, Germany) in 5 ml of phosphate buffer and the AChE activity was measured by the method of Ellman et al., (Ellman, Courtney, Andres, & Feather-Stone, 1961) using an ELISA microplate reader (Bio-Tek Instruments, ELx808). The absor-

Table 1. Animals weight during the course of study. Values are mean \pm SEM (n=7).

Treatment groups	Initial weight (g)	Final weight (g)
Control	205 \pm 5	238 \pm 6
Scopolamine	199 \pm 6	237 \pm 7
Piracetam	199 \pm 7	241 \pm 9
Walnut 2%	201 \pm 5	239 \pm 9
Walnut 6%	196 \pm 6	234 \pm 8
Walnut 9%	202 \pm 8	237 \pm 6

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balance was measured at 412 nm for a period of 10 min at intervals of 2 min. Second subgroups were fasted for 18 h and then their blood was collected and centrifuged to separate serum for estimation of total cholesterol and triglyceride at Razi laboratory (Kerman, Iran).

2.8. Statistical analysis method

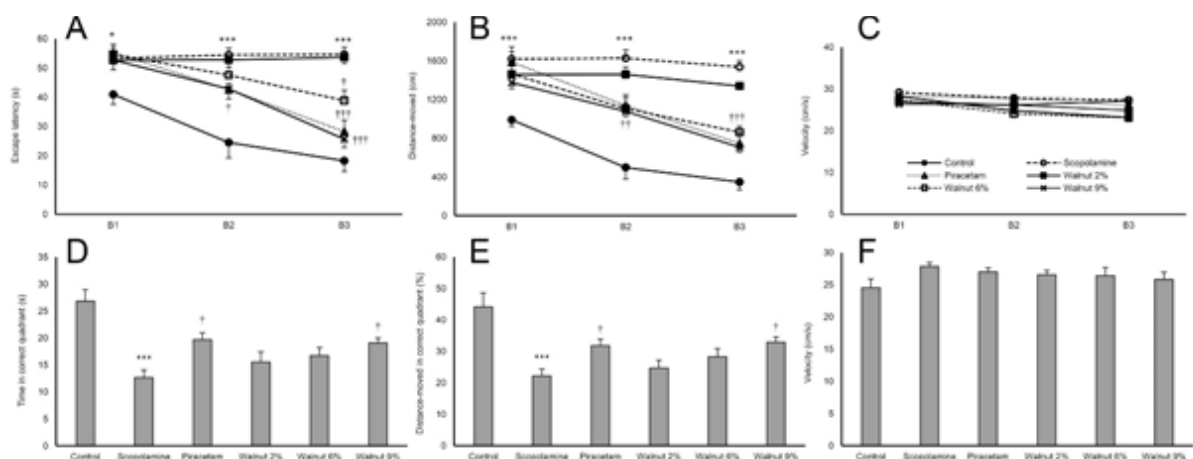
Data were analyzed using IBM SPSS statistics 21.0. One way ANOVA followed by Tukey test for post hoc analysis, was performed to compare the probe trial data in water maze, the passive avoidance number of shocks, the times on rod, the blood lipid profiles and the brain AChE activity. Kruskal–Wallis one-way analysis of variance was used to compare the passive avoidance latencies. Morris water maze latencies, velocities and body weight values were analyzed by repeated measure ANOVA. Differences with $P < 0.05$ between experimen-

tal groups at each point were considered statistically significant.

3. Results

3.1. Walnuts effect against the scopolamine-induced impairment in spatial learning and memory

No significant difference was observed in weight of animals during the course of the study (Table 1). The effects of walnuts consumption on spatial learning and memory was evaluated through the MWM test. Escape latencies and distances swum to the platform decreased over subsequent blocks of training in control, piracetam, walnut 6% and walnut 9% groups. Rats in these groups learned to find the hidden platform, and escape onto it. In contrast, scopolamine-induced memory impairment made escape latency and distance-moved of the scopolamine group significantly higher than that of the control group during all trial sessions (Figure 1A, 1B). Repeated measure ANOVA revealed no significant difference between escape latencies in block 1 of piracetam and walnuts-consumed groups compared to the scopolamine group. This parameter was significantly decreased in block 2 of piracetam and walnut 9% groups in comparison with the scopolamine group. Moreover, escape latencies of piracetam, walnut 6% and walnut 9% groups in block 3 significantly decreased compared to the scopolamine group (Figure 1A). As shown in Figure 1B, the distance-moved in block two and three was also decreased in the piracetam and walnuts-consumed groups compared to the scopolamine group. There were no significant differences in the swimming speeds among the groups during three blocks, indicating that the swimming speed did not



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Figure 1. Effects of walnuts consumption on memory impairments in the MWM test. Escape latency (A), distance-moved (B) and velocity (C) of the animals during training trial sessions; Time in the correct quadrant (D), distance-moved in the correct quadrant (E) and velocity (F) of the animals in the probe trial. Values are mean \pm SEM (n=7). * $P < 0.05$ and *** $P < 0.001$ in comparison with the control group, † $P < 0.05$, †† $P < 0.01$ and ††† $P < 0.001$ compared to the scopolamine group.

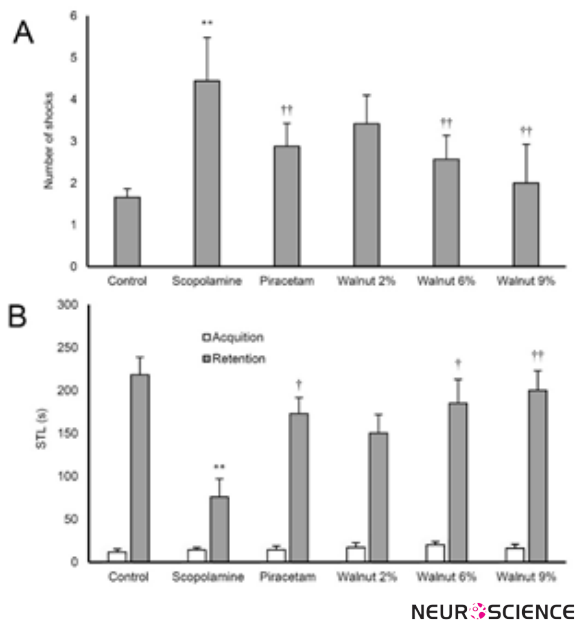


Figure 2. Effects of walnuts consumption on memory impairments in the passive avoidance task. Number of shocks (A) the rats of each group received and their step-through latencies (B). Values are mean ± SEM (n=7). ** P<0.01 in comparison with the control group, † P<0.05 and †† P<0.01 compared to the scopolamine group.

have any influence on the escape latency and distance-moved parameters (Figure 1C).

The results of the probe trial are shown in Figure 1D, 1E and 1F. One-way ANOVA and Tukey post hoc analysis indicated that piracetam and walnut 9% groups spent significantly more time and percentage of distance crossing over the platform site in the correct quadrant. No significant difference was observed between the mean velocities of the experimental groups during probe trial. The results of the visible test showed no significant difference among all groups (data not shown).

Table 2. Effect of walnuts consumption on serum lipid profiles. Values are mean ± SEM (n=7).

Treatment groups	Serum total cholesterol (mg/dl)	Serum triglycerides (mg/dl)
Control	62 ± 3.8	68.3 ± 6
Scopolamine	54.6 ± 4.1	70.3 ± 5.3
Piracetam	60.3 ± 3.3	70.1 ± 8.9
Walnut 2%	50.1 ± 3.9	53.6 ± 6.3
Walnut 6%	52.1 ± 1.7	57.6 ± 4.7
Walnut 9%	50 ± 2.3	55.1 ± 5.9

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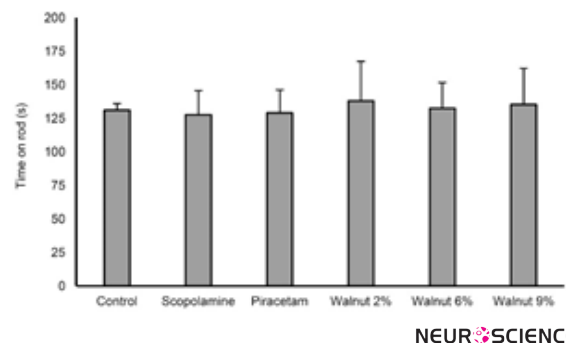


Figure 3. Effects of walnuts consumption on locomotor activity. Values are mean ± SEM (n=7).

3.2. Walnuts effect against the scopolamine-induced memory impairment in passive avoidance test

The passive avoidance test was also used to evaluate learning and memory. Figure 2A shows the effects of walnuts consumption on the mean number of shocks which the rats of each group received to remain in the light compartment for five consecutive minutes. The results showed that walnuts consumption in walnut 6% and walnuts 9% groups decreased the number of learning trials. The mean STL of scopolamine-treated group in the retention phase was significantly shorter than that of the control group (Figure 2B). Walnuts at a dose of 6% and 9% which were consumed for 4 weeks significantly increased the STL of retention phase and reversed scopolamine-induced amnesia.

3.3. Walnuts effect on locomotor activity

The results of the rotarod test showed no significant differences between different groups (Figure 3). This result indicates that the scopolamine, piracetam and walnuts did not affect the general locomotor activities of the rat.

3.4. Walnuts effect on serum lipid parameters and AChE activity of the brain

The obtained results showed that scopolamine significantly increased the AChE activity of brain in scopolamine group compared to the control ones. However, piracetam and walnuts consumption (6% and 9%) significantly decreased the AChE activity of the whole brain in comparison with the scopolamine group (Figure 4). Moreover, results showed no significant differences in the serum mean cholesterol and triglyceride levels (Table 2).

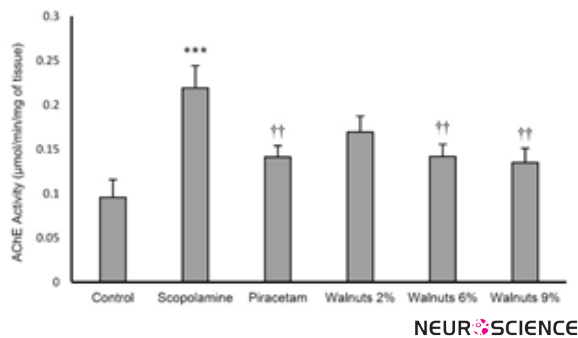


Figure 4. Effects of walnuts consumption on AChE activity of the whole brain. Values are mean \pm SEM (n=7). *** P<0.001 in comparison with the control group and †† P<0.01 compared to the scopolamine group.

4. Discussion

In the present study, the anti-amnesic effect of walnuts consumption on the scopolamine-induced memory impairment was evaluated using the MWM and passive avoidance tests. These tasks have been extensively used to measure learning and recall for evaluating the effects of aging, experimental lesions, drug effects and behavioral manipulations especially in rodents (Oh, Choi, Chang, & Park, 2009; Terry Jr, 2000).

Our obtained results showed that rats treated with scopolamine showed more prolonged escape latencies than rats from the control group in the MWM test. In contrast, a 4-week period consumption of walnuts showed some memory enhancing activity in the scopolamine-induced amnesic rats. Walnuts-consumed groups (walnut 6% and walnut 9%) showed lower scores in their latency and distance-moved parameters of MWM test which is an index of enhanced spatial memory of the animals (Morris, 1984). In case of the probe trial, consumption of walnuts in the walnut 9% group improved the memory of scopolamine-induced amnesic rats. This was revealed by an increase in the time and distance-moved of animals in the correct quadrant.

Similar results were obtained in the passive avoidance test. Pretreatment of rats with walnuts (6% and 9%) significantly decreased their training trials and in contrast, increased their step-through latencies of the retention phase compared to the scopolamine group. The similar step-through latencies of rats in the acquisition phase indicated the behavioral homogeneity of the animals.

Some recent studies challenge the viability of scopolamine as a cognitive impairer by questioning the relation of the alterations in the memory tasks to locomotor effects, instead of memory disruption (Klinkenberg &

Blokland, 2010). Our obtained results from the velocities of animals in the MWM test also the time animals remain on rod in the rotarod test showed no significant differences between locomotor activities of different groups. So these results demonstrate that walnuts consumption can improve learning and memory function of rats against scopolamine-induced amnesia. A recent study has shown that walnuts consumption could also improve memory in cisplatin-treated rats (Shabani et al., 2012).

The AChE activity of the brain was also evaluated to elucidate the underlying mechanisms of memory enhancing effects of walnuts. The level of ACh as the responsible neurotransmitter in conduction of electrical impulses between nerve cells is decreased in AD due to its rapid hydrolysis by AChE enzyme activity (Ladner & Lee, 1998). So AChE inhibitors could be considered as the main drugs for the management of AD. Our results showed that the scopolamine as a cholinergic receptor antagonist significantly increased the AChE activity of the brain compared to the control group and in contrast the walnuts (6% and 9%) consumed groups showed significantly lower AChE activities in comparison with the scopolamine group. Consumption of 2% daily walnuts neither improved the memory deficits nor decreased the AChE activity of the brain. Therefore, the anti-amnesic activity of walnuts could be attributed to the AChE activity and these nuts might have protective effects against AChE-related diseases, such as AD, by suppressing the AChE activity in nerve cells. Moreover, in a recent study, it was shown that walnut-supplemented diets could enhance the cholinergic transmission in the striatum of aged rats through increasing ACh synthesis or inhibiting AChE activity (Poulose, Bielinski, & Shukitt-Hale, 2012).

Other mechanisms could be also responsible for protective effects of these nuts. Haider et al., showed that walnuts consumption could enhance the memory function. They attributed these memory enhancing effects of walnuts to the increased 5-hydroxy tryptamine (5-HT) metabolism of the brain (Haider et al., 2011). Walnuts are rich source of tryptophan, the precursor of 5-HT (Brufau, Boatella, & Rafecas, 2006; Young & Gauthier, 1981) and as a result, walnuts consumption could increase the brain 5-HT function which might be involved in their memory enhancing effects. It has been also shown that walnuts as a good source of antioxidants could exert some protective effects against amyloid beta protein (A β)-mediated cell death by reducing the generation of free radicals, inhibiting membrane damage and attenuating DNA damage (Muthaiyah, Essa, Chauhan,

& Chauhan, 2011). Aggregation and fibrillization of A β is one of the major pathological features in AD (Masters et al., 1985).

Melatonin is another compound which could effectively reverse the memory and synaptic impairments in scopolamine-induced amnesic rats (Wang et al., 2013). Walnuts contain considerable amount of melatonin and consumption of these nuts could significantly increase the melatonin level and total antioxidant capacity of blood (Harandi et al., 2013; Reiter, Manchester, Tan, 2005). It has been shown that deficiency of this hormone could lead to cognitive impairment and dementia by degeneration of cholinergic neurons and deposition of some aggregated proteins in the brain (Lahiri, Chen, Lahiri, Bondy, & Greig, 2005). Interestingly a recent study showed that walnuts consumption could also contribute to increases in brain-derived nerve growth factor (BDNF) of humans and BDNF could prevent or reverse memory loss, cognitive impairment, brain cell degeneration and cell death in different models of AD and aging (Nagahara et al., 2009; Peng, Wu, Mufson, & Fahnestock, 2005).

Moreover, while most nuts contain monounsaturated fats, walnuts are well-known for their high level of polyunsaturated fatty acids (PUFAs) of which α -linoleic acid (C18: 3n-3, ALA) is more important. ALA as an omega-3 fatty acid is the precursor for eicosapentaenoic acid (C20: 5n-3, EPA) and docosahexaenoic acid (C22:6n-3, DHA). EPA modulates important inflammatory and immune functions while DHA is important for neural membrane stability, neuroplasticity, synaptic plasticity, gene expression, cell migration, apoptosis, signal transduction and neurotransmission (Joseph, Cole, Head, & Ingram, 2009). ALA is an essential PUFA which cannot be synthesized in the body, therefore it must be obtained from the diet and walnuts have the highest level of this omega-3 fatty acid compared to all other edible plants (Carey, Poulouse, & Shukitt-Hale, 2012). It should not be neglected that in our study, diets were not balanced for fat percentage and calories. However, no significant differences in serum lipid profiles were observed between different groups. Collectively, more studies seem to be needed to acquire a full understanding of the possible mechanisms responsible for the memory improving property of these nuts.

In conclusion, the present study demonstrates that walnuts have potent memory enhancing effects against scopolamine-induced amnesia in rats. These effects might result from the inhibition of AChE activity of the brain. However, the mechanism by which walnuts enhance

the memory and the active components responsible for these beneficial effects remain unclear and require further studies.

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