Dietary Supplementation of Walnuts Improves Memory Deficits and Learning Skills in Transgenic Mouse Model of Alzheimer’s Disease

Balu Muthaiyah, Musthafa M. Essa, Moon Lee, Ved Chauhan, Kulbir Kaur and Abha Chauhan
NYS Institute for Basic Research in Developmental Disabilities, Staten Island, NY, USA

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Abstract. Previous in vitro studies have shown that walnut extract can inhibit amyloid-β (Aβ) fibrillization, can solubilize its fibrils, and has a protective effect against Aβ-induced oxidative stress and cellular death. In this study, we analyzed the effect of dietary supplementation with walnuts on learning skills, memory, anxiety, locomotor activity, and motor coordination in the Tg2576 transgenic (tg) mouse model of Alzheimer’s disease (AD-tg). From the age of 4 months, the experimental groups of AD-tg mice were fed custom-mixed diets containing 6% walnuts (T6) or 9% walnuts (T9), i.e., equivalent to 1 or 1.5 oz, respectively, of walnuts per day in humans. The control groups, i.e., AD-tg and wild-type mice, were fed a diet without walnuts (T0, Wt). These experimental and control mice were examined at the ages of 13–14 months by Morris water maze (for spatial memory and learning ability), T maze (for position discrimination learning ability), rotarod (for psychomotor coordination), and elevated plus maze (for anxiety-related behavior). AD-tg mice on the control diet (T0) showed memory deficit, anxiety-related behavior, and severe impairment in spatial learning ability, position discrimination learning ability, and motor coordination compared to the Wt mice on the same diet. The AD-tg mice receiving the diets with 6% or 9% walnuts (T6 and T9) showed a significant improvement in memory, learning ability, anxiety, and motor development compared to the AD-tg mice on the control diet (T0). There was no statistically significant difference in behavioral performance between the T6/T9 mice on walnuts-enriched diets and the Wt group on the control diet. These findings suggest that dietary supplementation with walnuts may have a beneficial effect in reducing the risk, delaying the onset, or slowing the progression of, or preventing AD.

Keywords: Alzheimer’s disease, antioxidants, dementia, inflammation, oxidative stress, transgenic mice, walnuts

INTRODUCTION

Alzheimer’s disease (AD) is a neurodegenerative disease that causes the gradual loss of memory, language, and reasoning over a period of 5–20 years in elderly people. It is characterized by neuronal loss and by progressive accumulation of fibrillar amyloid-β (Aβ) as amyloid plaques, and of paired helical filaments as neurofibrillary tangles in the brain [1–3]. Extensive evidence suggests increased oxidative stress [4–7] and inflammation [5, 8, 9] as prominent features in AD that may be related causally to neuronal cell loss and dysfunction. The neuropathological changes associated with AD evolve gradually over decades before the clinical symptoms become evident in affected individuals [10–12]. Enhanced oxidative damage has also been reported in the early stages of mild cognitive impairment in the brains of individuals with AD, and in cerebrospinal fluid from individuals with early signs of dementia [7, 13, 14].

Several studies suggest that plant foods rich in phenols and flavonoids are an important class of defensive...
antioxidants and may provide health benefits [15]. Recently, potential benefits of dietary antioxidants have also been suggested in neurodegenerative diseases including AD. Plant extracts such as green tea, gingko biloba, and curcumin have been found to prevent oxidative stress–mediated apoptosis in cultured neurons and to reduce the oxidative stress that is associated with AD [16–19]. Recent evidence suggests that a diet with walnuts (Juglans regia L.) may also help to reduce the risk of developing age-related diseases because walnuts are rich in nutrients that have antioxidant and anti-inflammatory properties. The brain requires a sufficient amount of water, vitamins (such as folate, thiamine, vitamins B6, and B12), α-lipoic acid, lutein, and n-3 fatty acids [20]. Walnuts contain a number of potential neuroprotective compounds such as gamma tocopherol (vitamin E), folate, melatonin, flavonoids, and phenolic acid (ellagic acid), and a significant amount of n-3 α-linolenic acid (ALA) (a plant-based omega-3 fatty acid) [21–27]. When 1,113 different food items were analyzed for antioxidant content, walnuts ranked second [27].

In an in vitro study, walnut extract inhibited the fibrilization of synthetic Aβ, and solubilized the pre-formed Aβ fibrils [28]. Several in vitro studies have shown that Aβ exhibits cytotoxic properties by producing reactive oxygen species (ROS) and inducing oxidative stress [29–32]. We recently reported that walnut extract has a protective effect against Aβ-induced cell death by reducing ROS generation and oxidative stress, inhibiting membrane damage, and attenuating DNA damage [33].

Aβ is produced by the proteolytic cleavage of amyloid-β protein precursor (AβPP). AβPP-transgenic (tg) mice with the AβPP gene mutation show deposition of Aβ in the brain and memory deficit, and serve as an animal model of AD. The recommended daily serving of walnuts is one ounce (oz), i.e., 28 g, which equals one-quarter cup, or 12–14 walnut halves.

In this study, we examined the effect of dietary supplementation with 6% or 9% walnuts (equivalent to 1 oz and 1.5 oz, respectively, daily intake of walnuts in human) on the learning skills, memory, motor coordination, and anxiety-related behavior in a transgenic mouse model of AD.

**MATERIALS AND METHODS**

**Animals**

Transgenic (tg) female mice (B6: JIL-Tg (AβPPSWE) 2576Kha) with Swedish double mutations K670N and M671L of AβPP at the age of 11 weeks were purchased from Taconic Farms, Inc., Germantown, NY (hemizygous model # 001349-TF). Wild type (Wt) mice (model # 001349-WT) were also purchased from the same facility. These Wt mice were littermate controls that were produced in the same litters as the tg mice, but did not carry any manipulated gene of interest. Only animals with dark eye color (not pink-eyed) that tested negative for the Pde6bβ retinal degeneration mutation were included in the study. Because pink-eyed animals and retinal degeneration associated with the rd1 allele of the Pde6b gene can cause light sensitivity and/or blindness and can affect the results of behavioral tests, they were excluded from our studies. The mice were housed separately, one in each cage, and the body weight of each mouse was noted. They were maintained and cared for in our institute’s Animal Colony. They were given the autoclavable Taconic #31 diet for five weeks during a period of acclimatization. From the age of four months, mice were fed either a control diet without walnuts or a walnut-enriched diet. The AD-tg mice were randomly divided into different groups. All protocols were consistent with the National Institutes of Health Guidelines for the humane treatment of animals. The project was approved by the Institutional Animal Care and Use Committee of the NYS Institute for Basic Research in Developmental Disabilities.

**Diet**

English walnut kernels were obtained from the California Walnut Commission. AD-tg mice were randomly divided into three groups: T0, T6, and T9. AD-tg (T6, T9) mice were fed custom-mixed diets containing 6% (w/w) walnuts (AIN-93G + 6% walnuts) or 9% (w/w) walnuts (AIN-93G + 9% walnuts), respectively, from 4 months of age. These diets were custom-made in pellet form by Harlan Sprague Dawley Inc., Madison, WI. The TD.94045 (AIN-93G) control diet was modified by adding 6% or 9% (w/w) walnuts for the experimental diets. Nutritional data for the walnuts were derived from the USDA database. The control groups, AD-tg mice (T0) and wild-type mice (Wt), were fed the control diet (AIN-93G). Table 1 provides the composition of these diets. The diets for the experimental and control mice were comparable in the contents of protein (17.7%), carbohydrate (60%), and fat (17.2%), as well as the total calorie intake (protein: 18.8 Kcal %, carbohydrate: 63.9 Kcal %, and fat: 17.3 Kcal %) (Table 1).
Table 1
Composition of control diet without walnuts (AIN-93G) for wild-type (Wt) and AD-tg T0 mice, and of 6% or 9% (w/w) walnut-enriched diet for AD-tg T6 and T9 mice, respectively. All the diets were formulated to be iso-caloric and iso-nutrient, and custom-made in pellet form by Harlan Sprague Dawley Inc.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Control</th>
<th>6% Walnut</th>
<th>9% Walnut</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>20.0</td>
<td>18.95</td>
<td>18.43</td>
</tr>
<tr>
<td>L-cystine</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
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<td>Corn-starch</td>
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<td>38.35</td>
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<td>13.2</td>
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<tr>
<td>Sucrose</td>
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<td>10.0</td>
</tr>
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<td>3.1</td>
<td>1.15</td>
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<td>Mineral mix, AIN-93G-MX</td>
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<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>Vitamin mix, AIN-93-VX</td>
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<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Choline bitartrate</td>
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<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>TBHQ, antioxidant</td>
<td>0.0014</td>
<td>0.0014</td>
<td>0.0014</td>
</tr>
<tr>
<td>Ground walnuts (English)</td>
<td>–</td>
<td>6.0</td>
<td>9.0</td>
</tr>
<tr>
<td>Total protein</td>
<td>17.7%</td>
<td>17.7%</td>
<td>17.7%</td>
</tr>
<tr>
<td>Kcal</td>
<td>18.8%</td>
<td>18.8%</td>
<td>18.8%</td>
</tr>
<tr>
<td>Total carbohydrate</td>
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<td>60.0%</td>
</tr>
<tr>
<td>Kcal</td>
<td>63.9%</td>
<td>63.9%</td>
<td>63.9%</td>
</tr>
<tr>
<td>Total fat</td>
<td>7.2%</td>
<td>7.2%</td>
<td>7.2%</td>
</tr>
<tr>
<td>Kcal</td>
<td>17.2%</td>
<td>17.3%</td>
<td>17.3%</td>
</tr>
</tbody>
</table>

Behavior tests

The following behavioral tests were done at the age of 13–14 months in Wt (i.e., normal control) mice fed the control diet, in AD-tg mice on the control diet (T0), and in AD-tg mice on the experimental diet containing 6% walnuts (T6) or 9% walnuts (T9). A total of 37 mice were tested, which included 7 of Wt, 9 of T0, 11 of T6, and 10 of T9 mice. The effect of a diet with walnuts on motor coordination (rotarod), emotionality (elevated plus maze), perseveration (T-maze), and cognitive capacity (Morris water maze) was analyzed. The sequence of tests counterbalanced among the experimental groups in such a way that water escape–motivated tests (Morris, T-maze) were separated by a week of resting.

Rotarod

The motor coordination test was performed using a rotarod (LE8500, Panlab), which consisted of a rod (3 cm in diameter) with a rubber surface and four lanes of spindle, each 5 cm wide. All animals received two trials of pre-training on the rotarod without rotation. During training, the animal was positioned on the rod, with its rotating speed gradually accelerating from 5 to 40 RPM in 10 min. Each trial was terminated when the animal fell on a plastic surface 15 cm below the rod. The training consisted of four sessions, given on two consecutive days. The dependent variable was the duration during which the animal remained on the rotating rod.

Elevated plus maze (EPM)

Animals’ emotionality was evaluated using an EPM, in which they were challenged to exhibit two conflicting responses, one to seek safety in a closed arm, and the other to explore a salient arm. The EPM, constructed of wood and painted black, was 1 m from the floor and consisted of two open arms (5 x 30 cm) and two closed arms (5 x 30 x 15 cm), extending at a right angle from a center arena (5 x 5 cm). All mice were kept in their home cages and placed in an adjacent room of the EPM test room for 30 min of acclimatization. One mouse at a time was placed in the center of the EPM, facing the closed arm. The exploratory behavior of the mouse was video-recorded for 5 min. The frequency and duration of entry into the center, the closed arm, and the open arm were analyzed.

Water T-maze

The learning capacities of position discrimination and reversal were evaluated with a T-maze. The water maze was constructed of 5-mm clear Plexiglas, with a start box (20 x 6 x 24 cm) affixed at a right angle to two goal arms (18 x 6 x 24 cm), submerged in 10-cm deep opaque water. At the end of each arm was placed
an escape platform submerged in water in such a way that it would not be visible from the choice point. On the initial trial, each mouse was allowed to swim to and escape through either goal arm. Once the mouse reached the platform, it stayed there for 10 s before being removed and dried. This procedure was followed by discrimination training of two blocks of 10 massed trials a day with the platform placed in the opposite goal box of the initial preference. A correction procedure was employed, and errors were defined as the mouse entering its full body length into the incorrect arm or retreating back to the start arm. When the mouse was unable to reach the platform in 60 s, it was rescued. Eighteen errorless trials in a day (90%) were considered the criterion of learning. Once acquisition learning was completed, reversal training was given, with the platform placed in the arm opposite to the one originally trained. The escape latency and the number of errors were recorded.

Morris water maze

The ability to learn to locate a hidden escape platform and then to remember this location was evaluated in a Morris maze, a circular pool measuring 90 cm in diameter and 36 cm in height. Constructed of galvanized steel and painted white, it was filled with opaque, milk water (26 ± 1°C) to a depth of 20 cm. It was located in a testing room with distinctive distal visual cues including a door, a set of furniture, and fixed lighting sets around the walls. A clear Plexiglas escape platform, measuring 10 × 10 × 19 cm, was placed 24 cm from the pool wall. Its top surface, covered with a white towel, was 1 cm below the water level. A closed-circuit video-camera (Video Tracking System, San Diego Instruments) mounted on the ceiling 2 m above the pool monitored and recorded the time it took for each mouse to reach the platform, as well as the distance it swam.

Acquisition training consisted of a total of 25 trials, given as five spaced trials a day for five consecutive days. The platform was placed in a fixed position (designated as northeast), whereas the start position randomly alternated between three locations: north, south and west. Upon reaching the platform, the mouse was allowed to remain on the platform for 10 s before it was removed, dried with a towel, and placed in a dry cage under a heat lamp until the next trial. If a mouse was unable to escape within 3 min, it was gently guided to the platform. The inter-trial interval was about 10 min. A 60-s probe trial followed, during which the platform was removed from the maze, and the amount of time the mouse swam in each of the four quadrants of the pool was recorded. A reversal test was given after a two-day resting period, in which the platform location was moved 180° from the original position (i.e., southeast). The reversal test consisted of five trials a day for three days, for a total of 15 trials. The measure of performance was the time each mouse took to reach the escape platform, except for the probe trial, for which the percent distance travelled in the trained quadrant was analyzed.

Data analyses

The typical analysis consisted of a multi-way analysis of variance (ANOVA, using primarily Statistica 9.1 software), with genotype/treatment as the between-subject factors, and trial (nested under training days or conditions, if necessary) as the within-subjects factor. The ANOVAs were supplemented by pairwise multiple comparisons (the Tukey-Kramer test) between genotype/treatment groups.

RESULTS

In general, AD-tg mice on the control diet showed significant impairment in all behavior tests as compared to the Wt animals. A significant improvement was observed in AD-tg mice receiving a diet enriched with 6% walnuts (T6) or 9% walnuts (T9) as compared to T0 mice. T6 and T9 mice showed no significant changes as compared to the Wt mice.

Body weights

Figure 1 shows the body weight (mean ± SD) of the mice from the Wt, T0, T6, and T9 groups at the age of 4 months when the specific diets were initiated, at 9 months (after 5 months of feeding experimental diets), and at 13 months, when behavioral testing was done. At the ages of 4 and 9 months, there was no significant difference among the Wt, T0, T6, and T9 groups. At 13 months of age, the body weights of T0 and T6 mice were significantly less (p < 0.05) than those of Wt mice, but the body weights of T9 mice were similar to those of Wt mice, and they were significantly higher (p < 0.05) than the weights of T0 and T6 mice.

Mortality rate

During a period of acclimatization for five weeks, from the ages of 11 weeks to 4 months of age, before the experimental diets were started, a high mortality of 10.6% was observed in the AD-tg group. This high
mortality rate may be caused by stress due to changes in environmental conditions. The experimental diets with 6% or 9% walnuts (T6, T9) and the control diets (T0) were initiated at the age of 4 months. From 4 to 14 months of age, a strong survival rate was observed for AD-tg mice: during this 10-month period, the mortality was 2 of 11 (18.2%) in T0 mice, 1 of 12 (7.7%) in T6 mice, and 1 of 11 (8.3%) in T9 mice.

**Rotarod**

The performance on the Rotarod was analyzed using the duration of time the mice remained on the accelerating rod during the four sessions, which revealed a significant effect of genotype/treatment, $F_{3,33} = 4.539, p < 0.01$. A significant interaction between treatment and trial ($F_{3,99} = 2.1631, p < 0.05$) indicated differential rates of motor learning during the two-day sessions. Post-hoc analyses of trials 1 and 4 (Fig. 2) revealed that for trial 1, the durations of the four groups, i.e., Wt, T0, T6 and T9, were not significantly different from each other. On the last trial, however, the duration of T0 was significantly shorter than that of Wt mice, whereas the durations of T6 and T9 were indistinguishable from the duration of Wt. For within-group comparisons, T6, T9, and Wt recorded a significantly longer time in trial 4 than trial 1, whereas T0 showed no improvement. There was no significant difference in duration between T6 and T9.

**Elevated plus maze**

Responses to anxiety-producing environmental cues were evaluated by using the time spent for exploring in open arms of EPM assay, which is depicted in Fig. 3. There was a significant effect of diet with walnuts on total open arm activity, $F_{3,33} = 5.622, p = 0.01$. A significant interaction between genotype/treatment and exposure days ($F_{3,33} = 8.564, p < 0.001$) indicates that there were changes in responses during the two days of exposure. Post-hoc analyses revealed that all three AD-tg groups (T0, T6, T9) significantly reduced their open arm activity ($p < 0.5$) to the level of Wt animals, whereas T0 activity remained elevated, as compared to all three other groups ($p < 0.5$). The activity levels of the two groups of AD-tg mice on a diet with walnuts (T6, T9) were not different from each other on either day.
Fig. 4. Effect of diet with 6% or 9% walnuts on T-maze position discrimination learning by AD-tg mice. Data represent the total number of errors (mean ± SE) made during acquisition and reversal phases of T-maze learning by AD-tg (T0, T6, T9) and wild-type (Wt) mice.

**Water T-maze**

The total errors each group of mice made during the acquisition and reversal learning phases are presented in Fig. 4. A significant main effect ($F_{3, 33} = 4.654, p < 0.01$) indicated differential learning rates by the genotype/treatment groups. Post-hoc comparisons showed that T6 and T9 mice, while not different from the Wt animals, made significantly fewer errors than the T0 mice ($p < 0.05$) for acquisition. For reversal, although T0 mice showed higher error scores than others, no group differences reached a statistical difference. Analyses of the escape latency yielded similar results.

**Morris water maze**

Although all groups reduced their escape latency over the training period, only the T6 and T9 groups showed a significant reduction by the last day of learning ($p < 0.05$), whereas T0 mice failed to show any significant change. Figure 5A summarizes the total escape latency during the acquisition and reversal phases of learning, which indicated that the T0 mice were deficient in learning to locate the hidden platform. An ANOVA on the total escape latencies of acquisition and reversal phases resulted in a significant main effect, $F_{3, 33} = 3.283, p < 0.05$. Differential rates of learning were more clearly demonstrated in the probe trials. As shown in Fig. 5B, the T6 and T9 mice spent more time than the T0 mice in the area where the escape platform had been placed. An ANOVA on the activity in the trained quadrant after the acquisition and reversal trials showed a significant effect of diet with walnuts, $F_{3, 33} = 7.726, p < 0.01$. Post-hoc comparison tests revealed that T6, T9, and Wt mice had higher activity than T0 mice.

**DISCUSSION**

Previous *in vitro* studies have shown that walnut extract can inhibit the fibrillization of Aβ, solubilize Aβ fibrils [28], and protect cells against Aβ-induced oxidative stress and cell death [33]. In the present study, AD-tg mice at the ages of 13–14 months showed memory deficit, anxiety-related behavior, and severe impairment in (a) spatial learning, (b) position discrimination learning, and (c) motor coordination compared to the Wt mice that were fed a control diet. These behavioral alterations are similar to those reported for individuals with AD. Diets with 6% or 9% walnuts (equivalent to 1 oz or 1.5 oz walnuts per day in humans) ameliorated the memory loss, improved learning and
and apoptosis. It facilitates signal transduction and synaptic plasticity, gene expression, cell migration, and neuronal membrane stability, neuroplasticity, and inflammatory and immune functions.

Docosahexaenoic acid (DHA, 22:6n-3) is important for neuronal membrane stability, neuroplasticity, and cognitive function in the AD-tg mice in our study. While most nuts contain inflammatory effects of components in walnuts, walnuts are a nutrient-dense whole food that not only provide antioxidants (3.68 mmol/oz), such as flavonoids [15, 34, 35], ellagic acid [36], melanin [26, 37], gamma tocopherol, and selenium, but also ALA, the plant-based omega-3 fatty acid. These components of walnuts offer anti-inflammatory properties and protect brain cells from oxidative damage. In addition to antioxidants and ALA, walnuts are a good source of protein (4 g/oz) and fiber (2 g/oz). Walnuts also contain numerous other vitamins and minerals and provide magnesium (11% daily value) and phosphorus (10% daily value).

Walnuts ranked second among 1,113 different food items analyzed for their antioxidant content [27]. In another study, walnuts exhibited the best antioxidant properties when the antioxidant efficiency of different dry fruits was tested [38]. The highest phenolic content was reported in walnuts, followed by almonds and cashew nuts; and the lowest phenolic content was in raisins [38]. Another study examined the levels of antioxidants in different food items and reported at least 10 different antioxidants in walnuts. According to this study, 50 g of walnuts have significantly more antioxidants [302 mg gallic acid equivalent (GAE) of total phenols] than an 8-oz glass of apple juice (117 mg GAE in 240 ml), a milk chocolate bar (205 mg GAE in 1.5 oz), or a 5-oz glass of red wine (372 mg GAE in 150 ml) [23]. Therefore, we believe that diets enriched with walnuts may have provided improved memory and cognitive function in the AD-tg mice in our study because of the cumulative effects of walnuts’ ingredients to reduce oxidative stress and inflammation.

Walnuts have a high content of ALA that may be another contributing factor in improving the behavior abnormalities of AD-tg mice. While most nuts contain monounsaturated fats, only walnuts consist primarily of polyunsaturated fat (13 g of 18 g total fat in 1 oz of walnuts), of which ALA is 2.5 g. ALA is the precursor for eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3). DHA is important for neuronal membrane stability, neuroplasticity, synaptic plasticity, gene expression, cell migration, and apoptosis. It facilitates signal transduction and neurotransmission, and it increases serotonin and dopamine concentrations. EPA regulates the synthesis of arachidonic acid (20:4n-6) and modulates important inflammatory and immune functions [39, 40].

Diet supplemented with walnuts have been reported to have many other beneficial effects in health and disease. Numerous studies have suggested that the consumption of walnuts in the diet can reduce the risk of heart disease [41, 42], decrease total and low-density lipoprotein [42–47], increase total antioxidant capacity, and reduce plasma lipid peroxidation [48]. Animal studies have shown that a diet with walnuts slowed the growth of subcutaneously inoculated breast [49] and prostate cancer cells [50] in nude mice, and suppressed the development of spontaneous mammary gland tumors in transgenic mice [51]. A large cohort study of 83,818 women showed that consumption of 1 oz of nuts, such as walnuts, or of peanut butter five times or more each week reduced the risk of developing type 2 diabetes [52]. The beneficial use of walnut and its leaves has also been reported for the treatment of venous insufficiency and hemorrhoidal symptomatology, and for its anti-diarrheic, anti-microbial, anti- helmintic, depurative, and astringent properties [53–56]. The keratolytic, antifungal, hypoglycemic, hypotensive, and sedative activities of walnuts have also been reported [24, 54].

In an animal study, a diet with walnuts for 8 weeks improved cognitive and motor performance in aged rats (19 months old) [57]. Pribis et al. [58] examined the effects of a short-term (8 weeks) dietary supplementation with walnuts on cognitive performance in young adults. A significant increase was observed in inferential verbal reasoning with the walnut-supplemented diet. However, no significant difference was observed for mood, non-verbal reasoning, or memory, which may be because walnut supplementation in the diet was for only a few weeks. In the present study with a transgenic mouse model of AD, we fed the mice from 4 months of age with a 6% or 9% walnut-enriched diet for 9 to 10 months, and then analyzed its effect on memory and learning skills. Our findings suggest that long-term supplementation of a diet with 6% or 9% walnuts in AD-tg mice significantly reduced or prevented deficits in memory and learning compared to AD-tg mice on a diet without walnuts, and there was no statistically significant difference in the performance of these mice compared to wild-type control mice. We propose that early intervention with a diet with walnuts may help in reducing the risk of developing AD or delaying its onset because of the cumulative antioxidant and anti-inflammatory effects of components in walnuts.
ACKNOWLEDGMENTS

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