

Short Communication

Dose-dependent effects of walnuts on motor and cognitive function in aged rats

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Aged rats show decrements in performance on motor and cognitive tasks that require the use of spatial learning and memory. Previously we have shown that these deficits can be reversed by the polyphenolics in fruits and vegetables. Walnuts, which contain the *n*-3 fatty acids α -linolenic acid and linoleic acid, are a dietary source of polyphenols, antioxidants and lipids. Thus, the present study examined the effects of walnut supplementation on motor and cognitive ability in aged rats. Fischer 344 rats, aged 19 months, were fed a control, or a 2, 6 or 9% walnut diet for 8 weeks before motor and cognitive testing. Results for the motor testing showed that the 2% walnut diet improved performance on rod walking, while the 6% walnut diet improved performance on the medium plank walk; the higher dose of the 9% walnut diet did not improve psychomotor performance and on the large plank actually impaired performance. All of the walnut diets improved working memory in the Morris water maze, although the 9% diet showed impaired reference memory. These findings show for the first time that moderate dietary walnut supplementation can improve cognitive and motor performance in aged rats.

Polyphenols: Fatty acids: Antioxidants: Spatial memory: Learning

The ageing brain undergoes a myriad of changes resulting in alterations in neuronal functioning and ultimately degeneration of motor and cognitive abilities. In aged rodents, motor impairments manifest as poor performance on age-sensitive tests of balance and coordination^(1,2). Additionally, aged rodents exhibit impaired performance on the Morris water maze (MWM), which assesses spatial working memory⁽³⁾. These motor and cognitive impairments can be attributed to disruptions in neuronal functioning, including alterations in synaptic plasticity⁽¹⁾, decreased membrane fluidity⁽⁴⁾ and increased oxidative damage to DNA, lipids and proteins⁽²⁾. Because the deterioration of the ageing brain involves a number of intertwined processes, recent treatments to ameliorate age-related motor and cognitive dysfunction have focused on addressing several aspects of neurodegeneration at once. One promising avenue for the prevention and reversal of age-related motor and cognitive dysfunction is dietary intervention.

Dietary supplementation with a number of foods has been shown to induce a multiplicity of effects within the central nervous system, including vegetables, fruits and essential fatty acids from nuts and fish⁽⁵⁾. Whereas fish contain primarily the long-chain fatty acids EPA and DHA, nuts such as walnuts contain the *n*-6 and *n*-3 PUFA linoleic acid (LA; 18: 2*n*-6) and α -linolenic acid (ALA; 18: 3*n*-3)⁽⁶⁾. According to the US Department of Agriculture National Nutrient Database, 100 g walnuts contain approximately 38 g LA and 9 g ALA.

In addition to significant PUFA content, walnuts also contain a number of other potentially neuroprotective constituents including vitamin E, folate, melatonin and numerous antioxidant polyphenolics^(7,8). Melatonin and vitamin E have both been shown to improve cognitive performance in diabetic rats⁽⁹⁾, and polyphenols from several food sources administered through the diet can prevent and reverse behavioural and cognitive dysfunction in aged animals⁽¹⁰⁾. The present study was designed to examine the effects of dietary walnut supplementation on motor and cognitive functions in aged rats. We investigated three doses of walnuts (2, 6 or 9%) for efficacy in reversing age-related deficits in a battery of motor tasks and MWM performance (cognitive ability) in aged Fisher 344 rats. Fischer 344 rats, aged 19 months, were chosen for the present study because, by this age, the animals have already begun to exhibit decrements in motor and cognitive abilities^(3,11). Results indicate that moderate supplementation of aged rats with walnuts can reverse the effects of ageing on both motor and cognitive performance.

Methods

Animals

Seventy-three Fischer 344 rats (virgin males aged 19 months; Harlan Sprague Dawley, Indianapolis, IN, USA) were

Abbreviations: ALA, α -linolenic acid; LA, linoleic acid; MWM, Morris water maze.

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individually housed in viral antibody-free colony rooms subjected to monthly colony and vendor surveillance for health conditions, and maintained on a 12 h light–dark cycle. Rats were weight-matched and randomly assigned to one of four diet groups. In study 1, control and 2% walnut diets were fed (fourteen rats per group). In study 2, control and 6 and 9% walnut diets were fed (fifteen rats per group). Diets were provided *ad libitum* for 8 weeks. During the first study, two rats in the control group and one rat in the 2% diet group died, and two control rats were removed because of extensive weight loss and jaundice. For the second study, one control and one rat in the 6% group died, while two controls and one 9% rat were removed due to extensive weight loss and jaundice (final n 63). Weights were recorded throughout the study and food intake was assessed over a 72 h period. Animals were utilised in compliance with laws and regulations outlined in the National Institutes of Health (NIH), US Public Health Service (USPHS) Guide for the Care and Use of Laboratory Animals. These studies were approved by the Animal Care and Use Committee of our centre.

Diets

English walnuts (*Juglans regia*; California Walnut Commission, Sacramento, CA, USA) were ground with the skins on at Harlan Teklad (Madison, WI, USA). Walnuts were combined with the control diet (NIH-31); the amount of maize in the control diet was adjusted to compensate for the added volume of the walnuts, as we have done previously⁽²⁾. For example, the 6% walnut diet contained 60 g walnuts/kg and 40 g maize/kg; the control diet contained 100 g/kg maize. The percentage of dietary supplementation with walnuts was determined based on studies reporting a reduction in LDL-cholesterol in human subjects consuming walnuts⁽¹²⁾. A 6% walnut diet is approximately equivalent to a human eating 28 g (1 oz) walnuts/d; 9% is approximately equivalent to 42 g (1.5 oz).

Behavioural tests

Psychomotor. Age-sensitive tests of psychomotor behaviour⁽¹³⁾ were administered to animals during week 8 of treatment. Tests included: (1) rod walking, measuring psychomotor coordination and the integrity of the vestibular system by requiring the animal to balance on a stationary rod; (2) wire suspension, which measures muscle strength, an animal's ability to grasp a horizontal wire with its forepaws and to remain suspended; (3) plank walking, assessing balance and coordination by exposing the rats to three different sizes of horizontal planks; (4) inclined screen, which measures muscle tone, strength, stamina and balance by placing the animal on a wire mesh screen that is tilted 60°; (5) accelerating rotarod, which measures fine motor coordination, balance and resistance to fatigue by assessing the duration that a rat can remain standing or walking on a rotating, slowly accelerating rod.

Cognitive. Rats were tested on a MWM as previously described⁽³⁾. Briefly, the working memory version of the MWM was performed daily for four consecutive days during week 9 of the treatment, two trials each session, with a 10 min inter-trial interval between the two trials. At the beginning of each trial, the rat was gently immersed in the water at one of

four randomised start locations. Each rat was allowed 120 s to escape onto the platform; if the rat failed to escape within this time, it was guided to the platform. Once the rat reached the platform, it remained there for 15 s (trial 1; reference memory or acquisition trial). The rat was returned to its home cage between trials (10 min). Trial 2 (the working memory or retrieval trial) used the same platform location and start position as trial 1. Performances were videotaped and analysed with image tracking software (HVS Image, Hampton, UK), which allows measurements of latency to find the platform (s), path length (cm) and swimming speed (cm/s; path length/latency). Behavioural and cognitive testing was performed at approximately the same time every day during the animal's light period.

Statistical analyses

Since (a) the diets for both control groups were identical, and (b) the two control groups were not statistically different on any behavioural measures, they were combined to yield a single control group (n 22) for statistical analyses. For each behavioural measure, between-subjects ANOVA were performed using Systat (SPSS, Inc., Chicago, IL, USA) to test for statistical significance at the $P < 0.05$ level. Days or trials, when appropriate, were included in the model as a within-subjects variable. *Post hoc* comparisons to determine differences among groups were performed using Fisher's least significant difference (LSD) *post hoc* analysis.

Results

Groups exhibited no differences in weight during the course of the study (control group, 424 (SEM 5) to 421 (SEM 8) g; 2% walnut group, 424 (SEM 6) to 435 (SEM 5) g; 6% walnut group, 423 (SEM 7) to 429 (SEM 7) g; 9% walnut group, 420 (SEM 8) to 437 (SEM 8) g from start to end of study.) However, there were differences in food intake (control group, 20.9 (SEM 0.6) g/d; 2% walnut group, 20.0 (SEM 0.5) g/d; 6% walnut group, 21.5 (SEM 0.6) g/d; 9% walnut group, 22.9 (SEM 0.6) g/d), with the 9% walnut group eating significantly more than the control and the 2% group ($F(3, 58) = 3.63$; $P < 0.05$).

Motor testing

In the rod walking test, the 2% walnut diet improved performance (Fig. 1 (A)), as shown by a higher latency to fall compared with the control ($P < 0.05$) and 9% group ($P < 0.05$); the 6% walnut group was not different than any other group on this task. However, for the medium plank walk (Fig. 1 (B)) the 6% walnut group was significantly better than all the other groups ($P < 0.05$), having a higher latency to fall. On the large plank (Fig. 1 (C)), the 9% walnut group fell from the plank significantly quicker than the control and the 6% group ($P < 0.05$), showing impaired motor performance. No group differences were seen on the wire suspension, rotarod, inclined screen or small plank tests (data not shown). Therefore, both the 2% and 6% walnut-supplemented diets improved performance on motor tests that rely on balance, coordination and strength, while the 9% walnut diet actually impaired performance.

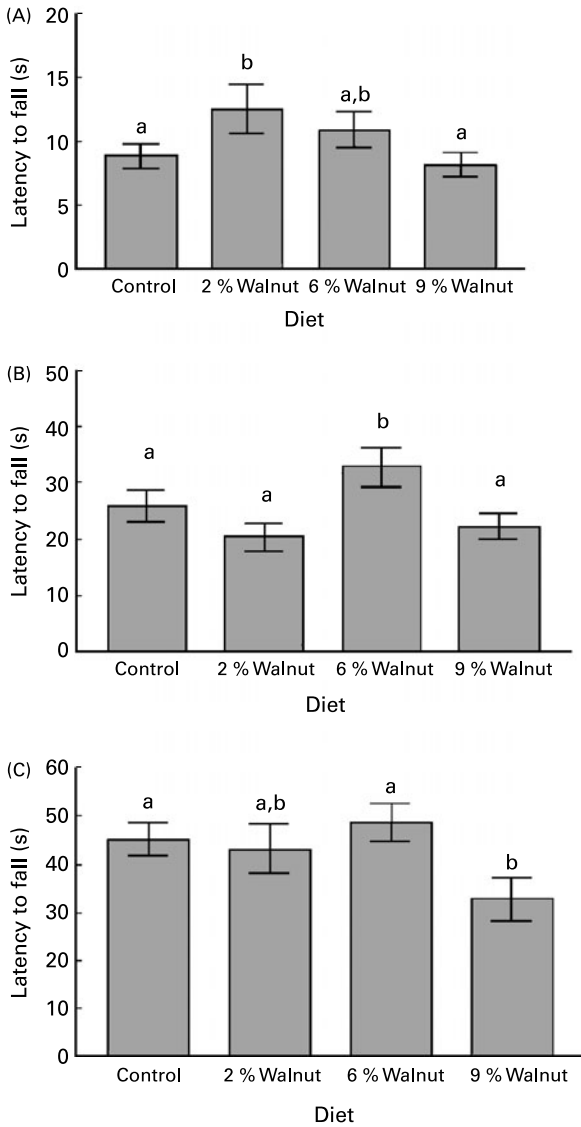


Fig. 1. Latency to fall (s) in the rod walking (A), medium plank (B) and large plank (C) tests for the control, 2, 6 and 9% walnut diet groups following 8 weeks of supplementation. Values are means, with standard errors represented by vertical bars. ^{a,b}Means with unlike letters are significantly different ($P < 0.05$; Fisher's least significant difference test).

Cognitive testing

All of the walnut diets improved working memory in the MWM, although the 9% diet showed impaired reference memory. Separate *t* tests were performed between the two trial latencies or distances for each group for days 3 and 4 (the days which rely more on memory than learning) in order to see if the different diet groups significantly improved performance from trial 1 to trial 2, an indication of improved working memory. The 2, 6 and 9% walnut diet groups all showed significant ($P < 0.05$) differences in both latency (Fig. 2 (A)) and swim distance (Fig. 2 (B)) to find the platform between trial 1 and trial 2, indicating that these rats demonstrated one-trial learning, even with the 10 min retention interval. This one-trial learning was not found in

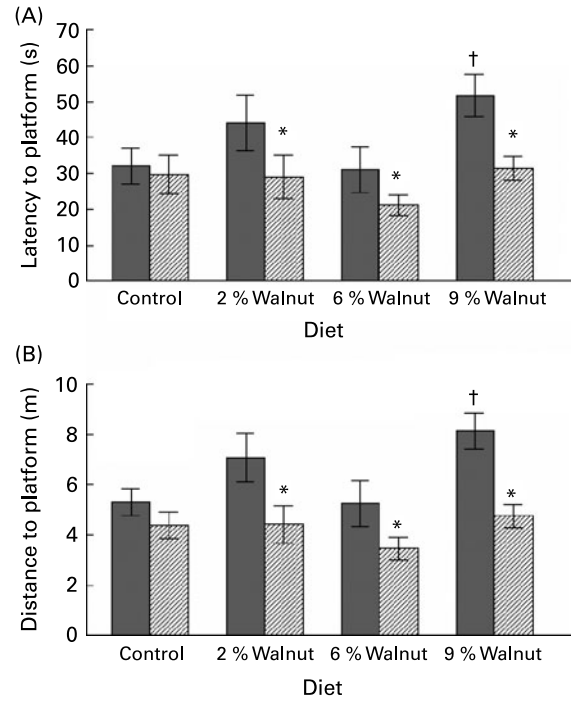


Fig. 2. Morris water maze performance assessed as latency (A) or distance (B) to find the hidden platform over days 3 and 4 of testing following 8 weeks of supplementation. (■), Trial 1; (▨), trial 2. Values are means, with standard errors represented by vertical bars. * Mean value was significantly lower than that for trial 1 ($P < 0.05$), indicating improved working memory in all walnut-supplemented groups. This improvement was not seen in the control rats. † Mean value was significantly different from that of the control group for the trial 1 performance ($P < 0.05$), indicating an impairment in reference memory for the 9% walnut group.

the control group. Even though the 9% walnut diet improved working memory, reference memory (trial 1) was impaired compared with control on both latency ($P < 0.05$; Fig. 2 (A)) and distance swam ($P < 0.01$; Fig. 2 (B)). This impairment could have accounted for the improvement in working memory in this group. These differences were not due to swim speed as there were no differences between the groups on this parameter.

Discussion

The primary findings in the present study are that diets containing 2 or 6% walnuts were able to reverse age-related motor and cognitive deficits as assessed by rod walk, plank walk and MWM tests. Animals on the 2% diet exhibited significantly improved motor performance on the rod walk test whereas animals on the 6% diet exhibited improved performance on the medium plank walk. Animals in all diet groups exhibited improved working memory performance on the MWM. However, a 9% dietary supplementation of walnuts resulted in large plank and reference memory impairments at this concentration. To our knowledge, this is the first study to demonstrate the beneficial effects of short-term walnut supplementation on motor and cognitive abilities in aged animals.

Although assessments of walnuts on motor and cognitive function are sparse, studies have demonstrated a positive

effect of PUFA on rodent behaviour. ALA and LA at a 1:4 ratio improved MWM performance in young rats⁽¹⁴⁾. Dietary ALA and LA supplementation has also been shown to impact on learning and memory in the senescence-accelerated (SAM) mouse, resulting in improved performance on the Sidman avoidance test and in light–dark discrimination learning⁽¹⁵⁾. Concomitant with an improvement in behaviour, animals also exhibited an increase in neuronal membrane fatty acid composition⁽¹⁶⁾. Since walnuts provide ALA and LA at the effective ratio of approximately 1:4, it is conceivable that walnut-derived PUFA could account for the effects of supplementation on motor and cognitive performance. Other constituents of walnuts such as vitamin E, melatonin, folate and polyphenols could also be contributing to the effects of moderate walnut supplementation^(7,17). Administration of vitamin E and melatonin will improve MWM performance in diabetic rats⁽⁹⁾, and melatonin has been shown to improve passive avoidance and elevated-plus maze performance in aged mice⁽¹⁸⁾. As with other nutritional substances, behavioural improvements may result from the consumption of polyphenols in walnuts. Walnut polyphenols include ellagitannins, which have been shown to inhibit oxidative stress and to modulate cell-signalling cascades⁽¹⁹⁾.

In the present study, although all doses of walnuts improved working memory, the 9% diet impaired reference memory in aged animals. The effects of walnut supplementation on MWM performance were therefore not dose-dependent, an effect which has previously been observed in studies exploring dietary supplementation with the PUFA DHA ethyl ester⁽²⁰⁾. When young mice supplemented with DHA ethyl ester at varying doses were tested for maze performance, it was found that although the highest dose of DHA ethyl ester was the most beneficial in improving maze performance, the relationship between DHA ethyl ester dose and maze performance was not linear⁽²⁰⁾. Similarly, in biochemical studies, the effects of fatty acids on arachidonic acid levels or lipid peroxidation were not dose-dependent^(21,22). In human subjects consuming EPA and DHA, no detrimental effects on erythrocyte lipid peroxidation were observed at low doses, but the opposite was true at higher doses of the dietary PUFA⁽²²⁾. Walnuts also contain a number of polyphenolic compounds that could be negatively affecting reference memory at a higher dose. In a previous study of Concord grape juice, our laboratory found that although supplementation of aged animals with a moderate amount (10%) of grape juice improved MWM performance, 50% supplementation did not improve spatial working memory⁽²³⁾. Dietary supplementation with polyphenol-rich foods may therefore induce not a linear effect but rather a U-shaped curve, with moderate doses imparting the most beneficial effect on cognition⁽²⁴⁾.

Considering the multitude of compounds within walnuts and the positive effects of moderate (6%) walnut consumption reported in the present paper, the specific cellular mechanisms of walnuts on neuronal functioning as related to behavioural and cognitive improvement are currently being examined in our laboratory. As is the case with a number of other nutritional substances, it is likely that walnuts provide a variety of bioactive substances that exhibit a multiplicity of effects on neural tissue to forestall and reverse motor and cognitive decline during the ageing process.

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