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ORIGINAL ARTICLE

Tocotrienol Rich Fraction Reverses Age-Related Deficits in Spatial Learning and Memory in Aged Rats

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Abstract Little is known about the effect of vitamin E on brain function. Therefore, in this study we evaluated the effect of tocotrienol rich fraction (TRF) on behavioral impairment and oxidative stress in aged rats. Thirty-six male Wistar rats (young: 3-months-old; aged: 21-months-old) were treated with either the control (olive oil) or TRF (200 mg/kg) for 3 months. Behavioral studies were performed using the open field test and Morris water maze (MWM) task. Blood was taken for assessment of DNA damage, plasma malondialdehyde (MDA) and vitamin E, and erythrocyte antioxidant enzyme activity. Brains were also collected to measure vitamin E levels. Results showed that aged rats exhibited reduced exploratory activity, enhanced anxiety and decreased spatial learning and memory compared with young rats. DNA damage and plasma MDA were increased, and vitamin E levels in plasma and brain were reduced in aged rats. Aged rats supplemented with TRF showed a markedly reduced level of anxiety, improved spatial learning and memory, reduced amount and severity of DNA damage, a reduced level of MDA, and increased levels of antioxidant enzyme activity and plasma/brain vitamin E compared with age-matched controls. In conclusion, TRF supplementation reverses spatial learning and memory decline and decreases oxidative stress in aged rats.

Keywords Tocotrienol rich fraction · Brain aging · Behavioural impairments · Spatial learning · Memory · Working memory · Open field test · Morris water maze task

Abbreviations

TRF	Tocotrienol rich fraction
MWM	Morris water maze
MDA	Malondialdehyde
DNA	Deoxyribonucleic acid
ROS	Reactive oxygen species
SOD	Superoxide dismutase
GPx	Glutathione peroxidase
CAT	Catalase
Hb	Hemoglobin
HPLC	High performance liquid chromatography

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Introduction

Aging is associated with a progressive decline in physical and cognitive functions. Various changes associated with the aging brain include a decrease in its volume and weight [1], neuronal loss, loss of dendritic spines [2] and neurochemical changes [3].

Oxidative stress is suggested to be involved in neurodegeneration. Reactive oxygen species (ROS) oxidize proteins, lipids and DNA, ultimately leading to cell death [4]. Elevated levels of the lipid peroxidation product,

malondialdehyde (MDA), have been shown to be associated with a decrease in visual-spatial memory and delayed declarative memory [5]. The brain is particularly vulnerable to ROS because of its high metabolic activity, high consumption of oxygen, low antioxidant enzyme levels and elevated content of polyunsaturated fatty acids [6].

Antioxidants scavenge ROS and may thus be used as neuroprotective agents. Fruits and vegetables are rich in antioxidants and their consumption has been shown to improve age-related decline in cognitive and motor functions [7]. *Ginkgo biloba* extracts have been reported to protect neurons particularly affected by age-related dementias, such as Alzheimer's disease [8]. Furthermore, a dietary supplementation of blueberry, spinach or spirulina is neuroprotective in a rat model of transient focal ischemic brain damage [9].

Members of the vitamin E family, tocopherols and tocotrienols, have been shown to exert neuroprotective effects alone or in combination under in vitro conditions [10, 11]. Supplementation of tocotrienol rich fraction (TRF) protects against oxidative DNA damage and improves circulating vitamin E levels in a rat model [12]. However, the effect of vitamin E on brain functions particularly during aging remains elusive. Data from human studies have been inconclusive. Of the nine published studies, seven have reported that vitamin E improves cognitive function in the elderly [13–19] and two have not observed any significant cognitive benefits [20, 21]. These discrepancies may be attributed to the differences in methods used to test behavioral impairments, the dose of supplements, the timing of study initiation, the antioxidant agent, and antioxidant sources. Most of these studies have focused on tocopherols alone. Mangialasche et al. [17] have reported that rather than the single isomers, a combination of the eight isomers of vitamin E (α, β, δ, γ -tocopherols and -tocotrienols) is associated with a reduced risk of AD. Therefore, in the present study, we investigated the effect of supplementing a vitamin E mixture containing tocopherols and tocotrienols but rich in tocotrienol (TRF) in aged rats compared with young rats. This mixture of vitamin E isomers was chosen, as tocotrienol had been shown to exert greater neuroprotective potential [22, 23]. We correlated the results with the level of oxidative stress markers such as DNA damage, plasma MDA and antioxidant enzyme levels.

Materials and Methods

Animals

Thirty-six male Wistar rats were fed with standard rat chow (Gold Coin, Malaysia) and tap water ad libitum. Animals were housed one animal per cage at room temperature under a 12 h dark:light cycle. This study was approved by the Universiti Kebangsaan Malaysia Animal Ethics Committee (protocol nos.: UKMAECFP/BIOK/2008/MUSAL-MAH/13-FEB/215-FEB-2008-OCT-2010). Every effort was made to limit the number of animals used and their suffering.

TRF Administration

Gold-Tri E70 (TRF) (Golden Hope Biorganic, Malaysia) consisted of approximately 149.2 mg/g α-tocopherol, 164.7 mg/g α-tocotrienol, 48.8 mg/g β-tocotrienol, 213.2 mg/g γ-tocotrienol and 171 mg/g δ-tocotrienol. Olive oil was bought commercially (Basso®, Italy).

Rats were randomly divided into two groups: young and old (3 and 21 months, respectively). Rats in both groups were given either TRF (200 mg/kg body weight, oral gavage) or control (equal volume of olive oil, oral gavage) ($n = 9$ per group) daily, for 3 months. At the end of the treatment period, the behavior of the rats was tested by the open field test and Morris water maze (MWM) task. Rats were then sacrificed and blood taken for determination of DNA damage, plasma vitamin E, antioxidant enzyme activity, and the levels of MDA. Brains were harvested, homogenized and analyzed for vitamin E.

Open Field

This test was used to evaluate locomotor activity, exploration and anxiety. A wooden box (all black) (60×60 cm^2) with 20 cm high walls was used. The floor was divided into 36 equal-sized squares (10×10 cm). Exploratory behavior was tested by placing rats into the open field chamber for 5 min and their behavior was recorded during the experiment. The number of fecal boli, number and duration of wall-supported rearing and grooming, number of squares traversed, and the number of central squares crossed were analyzed using the HVS Image 2100 tracking system (HVS Image, UK).

Morris Water Maze (MWM)

The MWM task was used to evaluate spatial learning and memory. More specifically, we carried out tests for reference memory and working memory. A black circular galvanized pool (140 cm diameter, 50 cm deep), filled with opaque water at 24 °C (±2 °C) to a depth of 30 cm was used. The pool was divided into four equal quadrants and a platform (13×13 cm) was placed in the center of one of the quadrants (target quadrant) 2 cm below the water surface. The laboratory light and several prominent visual features on the walls were held constant throughout the

reference memory task and were changed during the working memory task.

Reference Memory Task

For reference memory testing, we used the place navigation task (for spatial learning ability), the probe test (for spatial memory), and the cued navigation task.

Place Navigation During the task, rats were trained to find a hidden platform. The task was conducted on day 2 of the behavioral experiments (1 day after the open field test). It was carried out for 5 days and according to the method described by Liu [3], with some modifications. On the first day, the animals were trained to find the position of a fixed, hidden platform. A total number of six trials were conducted per day. Rats were placed in the water facing the wall of the tank, sequentially from four different entry points. Once the rat found and escaped onto the platform, it was given 20 s to allow for orientation to the visual cues in the room. Before proceeding to the next trial, the rat was rested for 20 s in a dry holding cage. The rat was gently guided on to the platform if it failed to locate the platform within 60 s. Rats were dried and returned to the cage at the end of training session. The tests were repeated for the next 4 days. The following parameters were recorded: escape latency (s), swim path (m) and swim speed (m/s).

Probe Test Probe test was conducted the day after completion of the place navigation experiment (i.e. day 7). In this test, the platform was removed. During the test, rats were released into the water at a fixed starting point and allowed to swim freely for 60 s. The starting point was the same for all animals. The percentage of time spent in the target quadrant and the number of platform crossings were measured.

Cued Navigation Cued navigation test was carried out on the day after completion of the probe test (i.e. day 8). During this test, the platform was shifted to another quadrant and raised 2 cm above the water surface. The edge of the platform was marked by a masking tape to make it more visible. All rats received six trials for 1 day. Again, the maximum searching time was 60 s and the rats were allowed to remain on the platform for 20 s.

Working Memory Task

The working memory test was carried out over the next 2 days. Rats were given two trials per day. The location of the hidden platform was changed between each trial. Each trial consisted of sample phase and test phase. The sample phase involved the first time in each trial that the rat was placed in the water tank. The rats rested for 1 or 60 min before they were re-tested (i.e. the test phase). The starting point for each phase was different. The maximum time for rats to search the hidden platform was 60 s and the rats were allowed to remain on the platform for 20 s before being removed.

Oxidative DNA Damage

Oxidative DNA damage was measured by the comet assay, as described by Singh et al. [24], but with slight modifications. Briefly, a thin layer of 0.6 % normal melting point agarose (Sigma-Aldrich, USA) was placed on fully frosted microscope slides and left at room temperature for 10 min. Whole blood (5 µl) mixed with 0.6 % low melting point agarose (Sigma-Aldrich, USA) was then spread on to the microscope slide. The slide was then immersed in cold lysing solution (2.5 M NaCl, 100 mM $Na_2EDTA\text{-}2H_2O$, 10 mM Tris, 1 % sodium N-lauroylsarcocinate, 1 % Triton X-100, 10 % dimethylsulfoxide, pH 10) at 4 °C for 1 h. The slide was then placed in electrophoresis buffer (0.3 M NaOH, 1 mM Na_2EDTA) for 20 min to unwind the DNA. Electrophoresis at 1–10 °C, 25 V, and 300 mA for 20 min then followed. The slide was then neutralized with 0.4 M Triz base buffer (pH 7.5). This procedure was repeated twice. The slide was then drained and 30 µl ethidium bromide (20 µg/ml) (Sigma-Aldrich, USA) was added; then the slide was covered with a new coverslip and placed in a humidified air-tight container in a refrigerator. The slide was examined at 40× magnifications using a fluorescence microscope (AxioCam MRC, Carl Zeiss, Germany) where photomicrographs of 150 randomly selected non-overlapping cells on each slide were made. Comet score software (TriTek Comet Score® Sumerduck, VA, USA) was used to determine the DNA damage and the severity of damage as a percentage of DNA in tail and tail moment.

Lipid Peroxidation

Determination of plasma MDA levels was carried out as described by Sim et al. [25]. This test is based on the derivatization of MDA with 2,4-dinitrophenylhydrazine (DNPH) and its conversion into pyrazole and hydrazone derivatives, which are separated via high performance liquid chromatography (HPLC). HPLC analyses were performed on a LC-10 AT VP® SHIMADZU (Kyoto, Japan) liquid chromatography system with a photodiode array detector and an auto injection valve. An alpha bond C18 125 A column (3.9 × 150 mm) with a 5 µm particle size (Alltech, Deerfield, USA) and a Shidmadzu Class-VP software system for data processing were used. MDA levels were expressed as nmol/l from calibration curves.

1,1,3,3-tetraethoxypropane (TEP) (Sigma-Aldrich, USA) was used as the MDA standard. The mobile phase was prepared with a mixture of 0.2 % (v/v) acetic acid in milli-Q water and acetonitrile (62:38) (v/v) at a flow rate of 1.5 ml/min at room temperature. Chromatograms were acquired at 310 nm. Briefly, 0.05 ml of plasma was added to 0.2 ml of 1.3 M NaOH and incubated in the water bath (60 °C) for 1 h. After incubation, the contents were mixed with 0.1 ml of 35 % perchloric acid and centrifuged at 10,000×*g* for 10 min (4 °C). Supernatant (0.3 ml) was then added to 12.5 ml of 5 mM DNPH and left for 30 min in the dark before detection using HPLC at 1.5 ml/min flow rate.

Antioxidant Enzyme Activities

The activity of superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) in erythrocytes were determined using commercial ELISA kits (Cayman, USA).

Vitamin E Determination

Extraction of Vitamin E

Brain tissues were weighed, homogenized in 0.1 M Triz-HCl (pH 7.5) at 4 °C and then centrifuged at 10,000×*g* for 10 min at 4 °C. The supernatants (100 µl) were treated with butylated hydroxytoluene (10 mg/ml) in 95 % ethanol, followed by 500 µl of 100 % ethanol. The mixtures were then centrifuged at 3,000×*g* for 15 min at 18 °C and the pellets discarded. The supernatants were then mixed with 1.5 ml hexane (Merck, Germany) and then centrifuged at 3,000×*g* for 15 min at 18 °C. The top layers (2.5 ml) were transferred to plain tubes and vacuum-dried (Heto Lab Equipment, Denmark), and then stored at − 20 °C until further analysis. Plasma samples were processed similarly as described above, except that 200 µl plasma samples were mixed with 10 mg/ml butylated hydroxytoluene. Prior to HPLC analysis, the samples were prepared with HPLC grade-hexane (1:100).

HPLC Analysis

The HPLC system (LC-10AT) consisted of SCL-10A system controller and SIL-10A auto injector with sample cooler (Shimadzu, Japan). The stationary phase was a 250 mm all-sphere five silica normal phase column, internal diameter 4.6 mm and particle size 5 µm. The mobile phase consisted of hexane:isopropanol (99:1) and was delivered at 1.5 ml/min. Eluted peaks were detected by the spectrofluorometric detector, RF-10AXL, and set at 294 nm (excitation wavelength) and 330 nm (emission wavelength). The vitamin E isomers were separated between 4 and 10 min. Peaks were integrated with LC workstation VP CLASS software (Shimadzu, Japan) and their concentrations were expressed in µg/ml. Sample peaks were obtained and compared with the standard. The concentrations of total vitamin E, α-tocopherol (ATF), α-tocotrienol (ATT), β-tocotrienol (BTT), γ-tocotrienol (GTT) and δ-tocotrienol (DTT) were presented in µg/ml.

Statistical Analysis

All data are expressed as the mean ± SEM. For each task in cognitive function test, data files created by the HVS software (HVS Image, UK) were exported to Microsoft Excel and SPSS (v. 16) for analysis. Statistical differences among more than three groups were estimated by a one-way ANOVA. For the water maze test, general linear model procedure (GLM) repeated measures ANOVA was performed to assess the overall differences for each behavior between the groups on day 1 until day 5 of training. When a significant interaction in the between-subjects variables (treatment and time) was determined, a subsequent one-way ANOVA was performed. In all statistical comparisons described below, p values less than 0.05 were considered significant.

Results

The Open Field Test

Aged rats produced significantly ($p < 0.05$) more fecal boli (4.0 ± 0.58) compared to young rats (1.2 ± 0.36) (Fig. 1a). Rearing in aged rats was significantly ($p < 0.05$) lower (6.55 ± 0.58) than in young rats (18.0 ± 1.44) (Fig. 1b). The total time spent grooming between all groups was not significantly different (Fig. 1c). The number of squares traversed in aged rats was significantly ($p < 0.05$) lower (99.22 ± 8.45) compared to young rats (137.56 ± 8.39) (Fig. 1d). Activity in the center zone was significantly ($p < 0.05$) lower (1.67 ± 0.60) compared to young rats (9.56 ± 1.31) (Fig. 1e). Aged rats supplemented with TRF excreted significantly ($p < 0.05$) lower fecal boli (2.0 ± 0.29) compared with age-matched controls (4.0 ± 0.58) (Fig. 1a). Furthermore, supplemented aged rats displayed significantly ($p < 0.05$) higher activity (5.56 + 1.57) in the center squares than age-matched controls (1.67 + 0.60) (Fig. 1e).

The MWM Task

In the place navigation test, the time to reach the hidden platform was significantly ($p < 0.05$) higher (32.48 ± 5.6 s) in aged rats compared to young rats (9.99 ± 0.8 s) on the

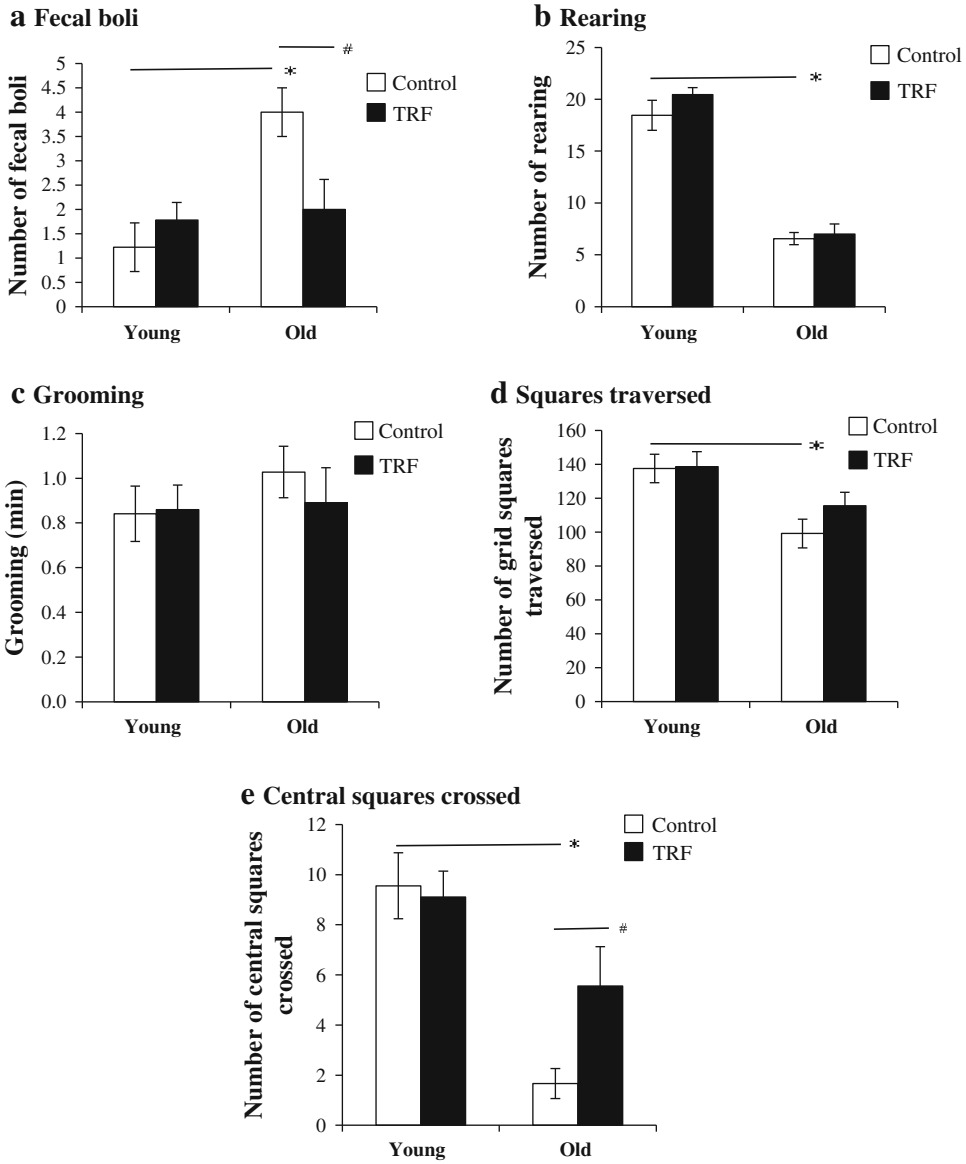


Fig. 1 Open field parameters as measured by **a** number of fecal boli, **b** number of rearing, **c** total time spent grooming in minutes, **d** number of grid squares traversed and **e** activity in center zone by young and old rats with and without tocotrienol rich fraction (TRF) supplementation. *A significant difference between young control vs. old control ($p < 0.05$). #A significant difference between old control vs. old TRF ($p < 0.05$)

fifth day of training (Fig. 2a). The time taken to reach the platform was significantly ($p < 0.05$) reduced in aged rats supplemented with TRF (19.98 ± 3.5 s). The distance to reach the platform was significantly ($p < 0.05$) longer (4.99 + 0.69 m) in aged rats compared to young rats (1.31 ± 0.12 m) on the fifth training day (Fig. 2b). However, distance travelled in aged rats supplemented with TRF was markedly reduced (3.58 ± 0.56 m) compared to age-matched controls (4.99 + 0.69 m). The swim speed remained unchanged between all groups over the 5 days of training (Fig. 2c).

Aged rats spent significantly ($p < 0.05$) less time (35.62 ± 2.54) in the target quadrants compared with young rats (49.03 ± 2.03 %) in the probe test (Fig. 3a). The time spent in the target quadrant of aged rats supplemented with TRF was not significantly different (39.20 ± 3.30) compared with age-matched controls (35.62 ± 2.54 %). In the probe test, the number of platform crossings in aged rats was significantly ($p < 0.05$) lower (1.78 ± 0.60) compared with young rats (6.56 ± 0.56) (Fig. 3b). The number of platform crossings was significantly ($p < 0.05$) higher (3.89 ± 0.45) in aged rats supplemented with TRF compared with age-matched controls (1.78 ± 0.60).

In the cued navigation task, the escape latency was significantly ($p < 0.05$) decreased over the six trials, but no significant differences were detected between the groups (Fig. 4a).

In the working memory test, the path length to reach the platform was significantly ($p < 0.05$) longer in aged rats (6.56 ± 0.90 m) compared with young rats (3.17 ± 0.77 m) in both the sample and test phase (Fig. 5a). The path length in the test phase was significantly ($p < 0.05$) shorter (3.53 ± 0.53 m) in aged rats supplemented with

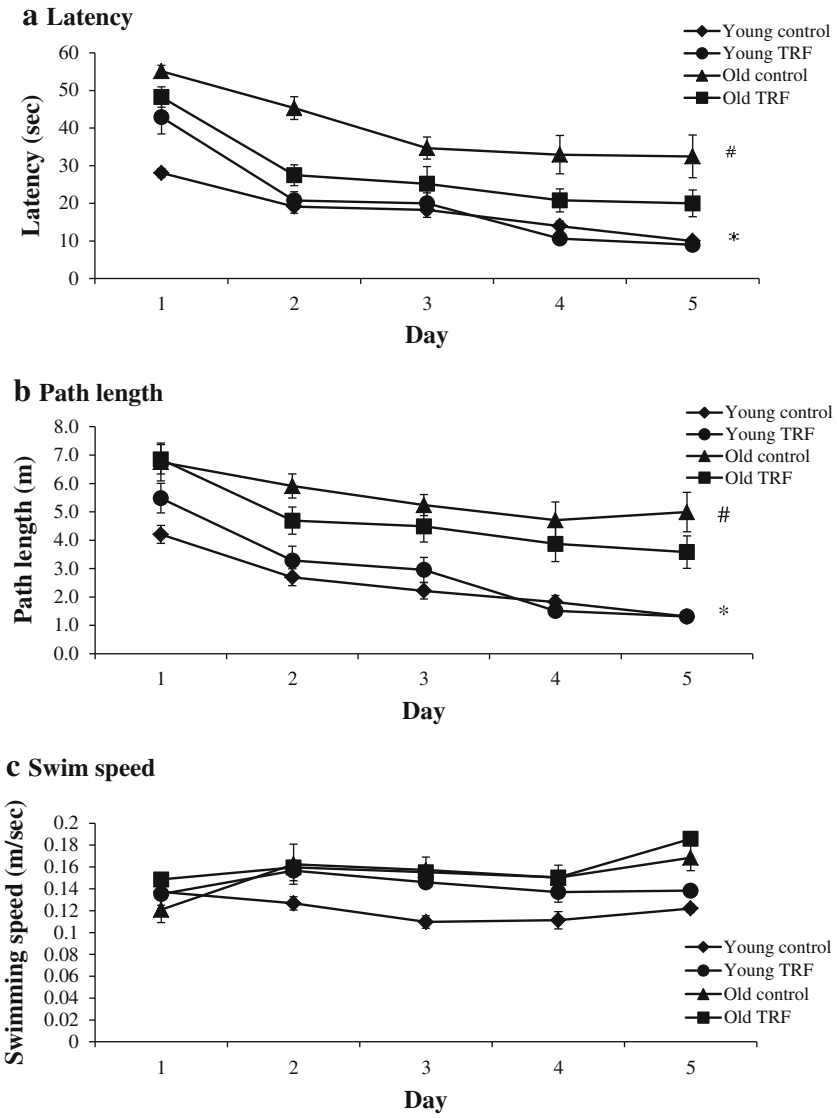


Fig. 2 Place navigation parameters as measured by **a** time taken to reach the platform at fixed location (latency), **b** distance traveled to the target platform, **c** swim speed in young and old rats with and without tocotrienol rich fraction (TRF) supplementation. *A significant difference between young control vs. old control ($p < 0.05$). # A significant difference between old control vs. old TRF ($p < 0.05$)

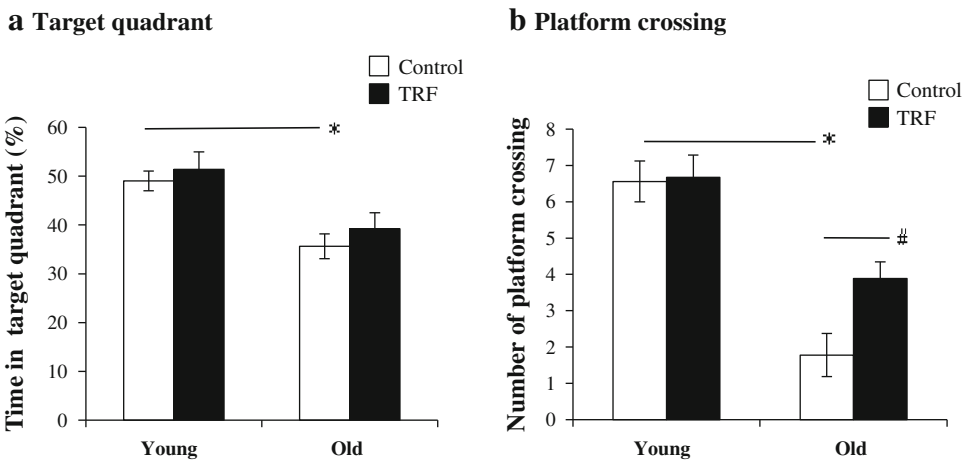


Fig. 3 Probe test parameters as measured by **a** percentage of time spent in target quadrant, **b** numbers of platform crossing in the target quadrant. *A significant difference between young control vs. old control ($p < 0.05$). # A significant difference between old control vs. old TRF ($p < 0.05$)

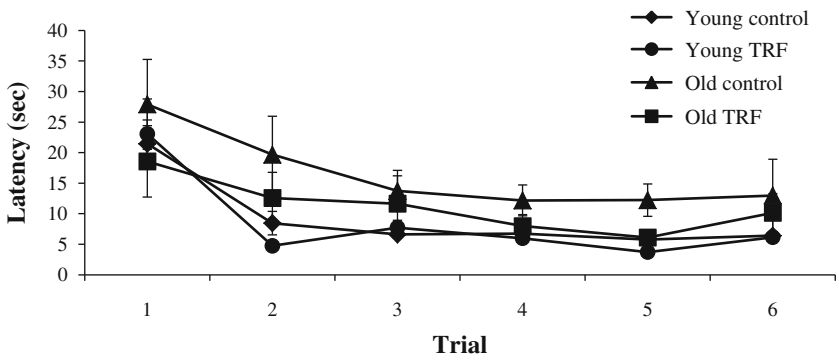


Fig. 4 Cued navigation parameter as measured by the latency to platform in young and old rats with and without tocotrienol rich fraction (TRF) supplementation

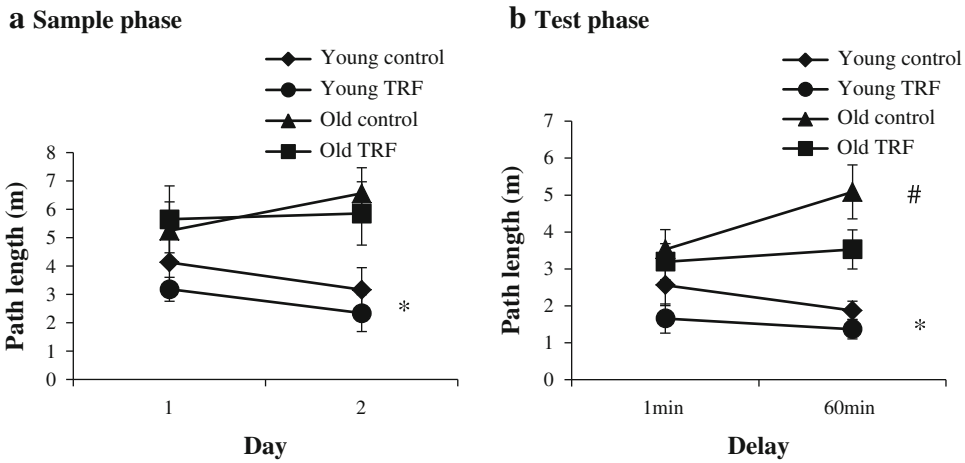


Fig. 5 Working memory tasks as measured by the path length (m) during **a** sample phase and **b** test phase. *A significant difference between young control vs. old control ($p < 0.05$). #A significant difference between old control vs. old TRF ($p < 0.05$)

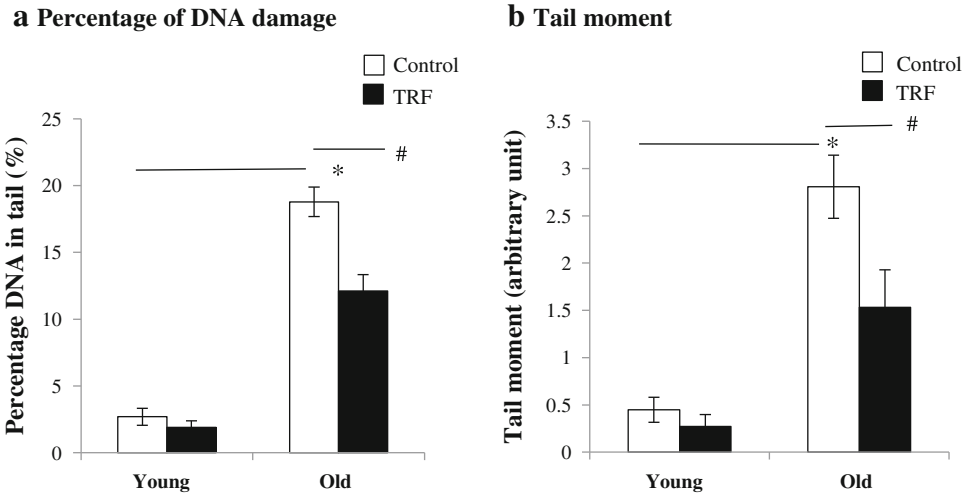


Fig. 6 DNA damage as measured by **a** the percentage of DNA in tail and **b** severity of DNA damage. *A significant difference between young control vs. old control ($p < 0.05$). #A significant difference between old control vs. old TRF ($p < 0.05$)

TRF compared to age-matched controls (5.08 ± 0.73 m) (Fig. 5b).

DNA Damage

The percentage of DNA damage was significantly ($p < 0.05$) increased (18.78 ± 1.10 %) in aged rats compared to young rats (2.7 ± 0.63 %) (Fig. 6). Similarly, the severity of DNA damage was significantly ($p < 0.05$) increased (2.81 ± 0.33 arbitrary unit) in aged rats compared with young rats (0.45 ± 0.13 arbitrary unit). The percentage of DNA damage was markedly ($p < 0.05$) reduced (12.11 ± 1.21 %) in aged rats supplemented with TRF compared with age-matched controls (18.78 ± 1.10). Similarly, the severity of DNA damage was significantly ($p > 0.05$) reduced (1.53 ± 0.40 arbitrary unit) in aged rats

supplemented with TRF compared with age-matched controls (2.81 ± 0.33 arbitrary unit).

Lipid Peroxidation

Plasma MDA levels were significantly ($p < 0.05$) increased (1.57 ± 0.05 nmol/ml) in aged rats compared with young rats (0.77 ± 0.04 nmol/ml) (Fig. 7). TRF supplementation in aged rats markedly ($p < 0.05$) reduced (1.29 ± 0.05 nmol/ml) MDA levels compared with age-matched controls (1.57 ± 0.05 nmol/ml). MDA levels remained unchanged in young rats supplemented with TRF versus age-matched controls.

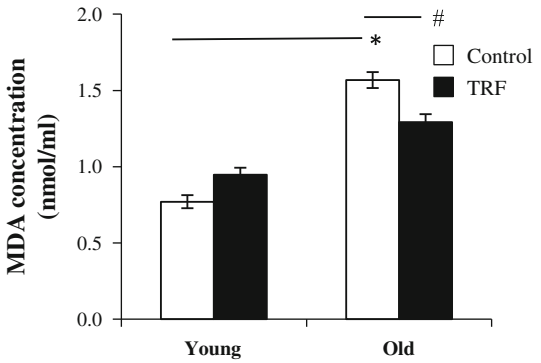


Fig. 7 Plasma malondialdehyde (MDA) level as measured by high performance liquid chromatography (HPLC) in young and old rats with and without tocotrienol rich fraction (TRF) supplementation. *A significant difference between young control vs. old control ($p < 0.05$). #A significant difference between old control vs. old TRF ($p < 0.05$)

Antioxidant Enzyme Activities

SOD activity was markedly ($p < 0.05$) decreased (3.14 ± 0.14 U/mg Hb) in aged rats compared with young rats (3.69 ± 0.17U/mg Hb) (Fig. 8). However, CAT activity was significantly ($p < 0.05$) increased (189.48 ± 15.56 nmol/min/mg Hb) in aged rats compared with young rats (83.75 ± 11.42 nmol/min/mg Hb). GPx activity remained unchanged between the two groups. Activity for all three enzymes (SOD, CAT and GPx) was significantly ($p < 0.05$) increased (4.03 ± 0.3 U/mg Hb, 255 ± 16.5 nmol/min/mg Hb, and 94.32 ± 13.1 nmol/min/mg Hb, respectively) in aged rats supplemented with TRF compared with age-matched controls.

Vitamin E Levels

Plasma vitamin E levels were significantly (Fig. 9) ($p < 0.05$) lower (10.11 ± 0.35 μg/ml) in aged rats compared with young rats (13.04 ± 0.47 μg/ml). Brain vitamin E levels were markedly ($p < 0.05$) lower (10.21 ± 0.22 μg/ml) in aged rats compared with young rats (10.87 ± 0.2 μg/ml). Plasma

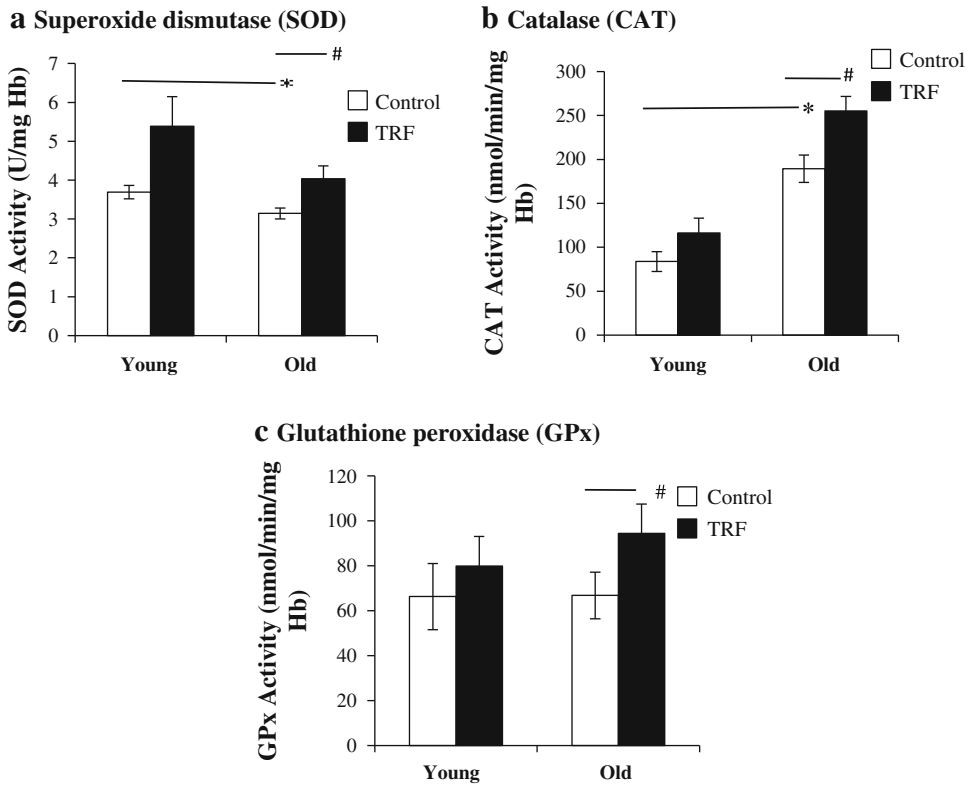


Fig. 8 Antioxidant enzymes activities **a** Superoxide dismutase (SOD), **b** catalase (CAT) and **c** Glutathione peroxidase (GPx) in young and old rats with and without tocotrienol rich fraction (TRF) supplementation. *A significant difference between young control vs. old control ($p < 0.05$). #A significant difference between old control vs. old TRF ($p < 0.05$)

levels of α-tocopherol in aged rats were significantly ($p < 0.05$) lower (7.58 ± 0.21 μg/ml) compared with young rats (11.70 ± 0.44 μg/ml). Brain levels of γ-tocotrienol were significantly ($p < 0.05$) lower (0.347 ± 0.03 μg/ml) compared with young rats (0.48 ± 0.08 μg/ml). Brain levels of δ-tocotrienol were markedly ($p < 0.05$) lower (0.625 ± 0.02 μg/ml) in aged rats compared with young rats (0.685 ± 0.02 μg/ml). Supplementation with TRF increased the levels of plasma vitamin E, α-tocopherol, and α-tocotrienol (16.71 ± 0.76 μg/ml, 12.10 ± 0.67 μg/ml, 2.34 ± 0.25 μg/ml, respectively) compared with age-matched controls (16.94 ± 0.84 μg/ml, 13.99 ± 0.72 μg/ml, and 0.93 ± 0.2 μg/ml, respectively). However, brain α-tocotrienol was increased only in aged rats (0.95 ± 0.04 μg/ml).

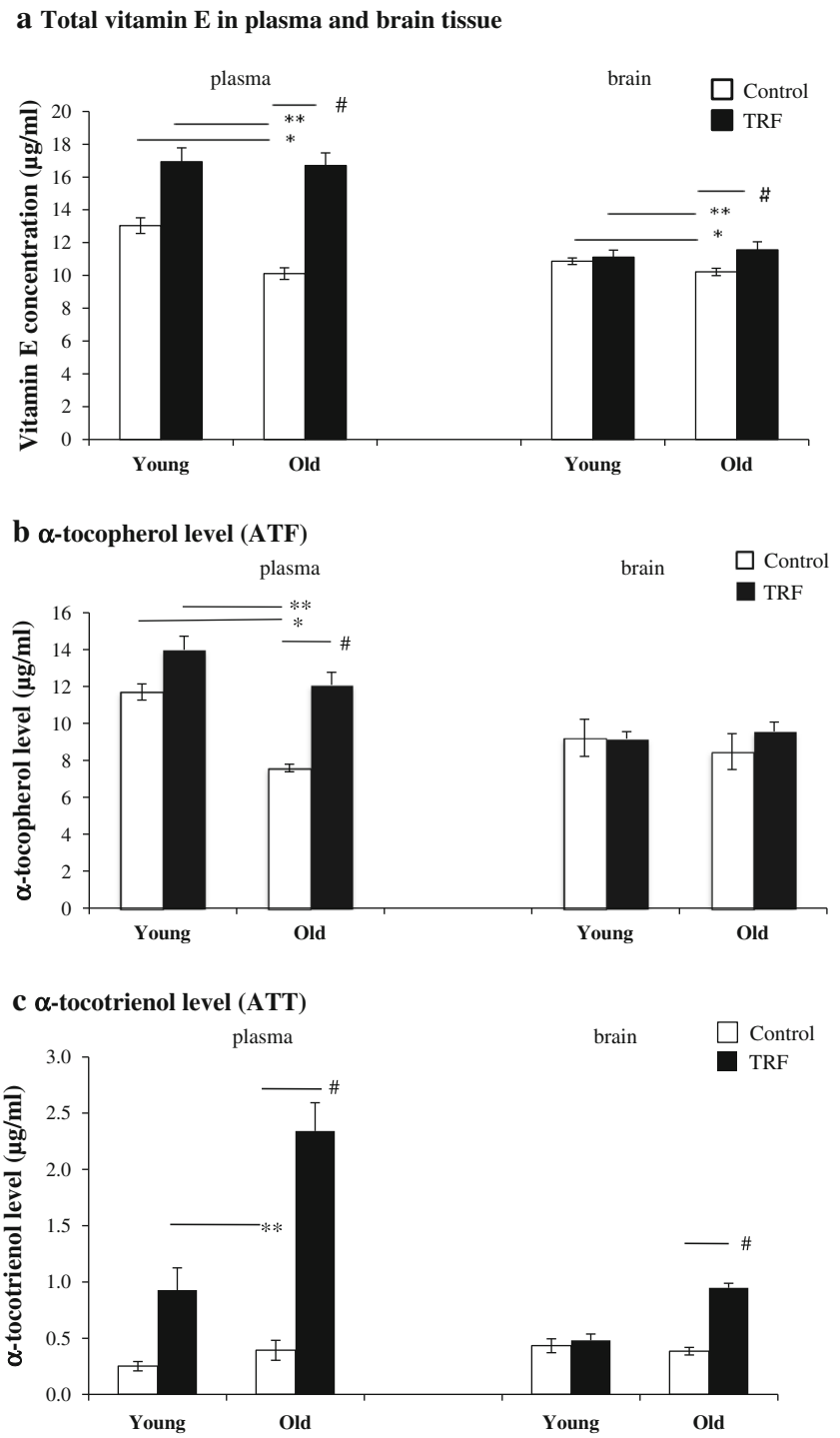
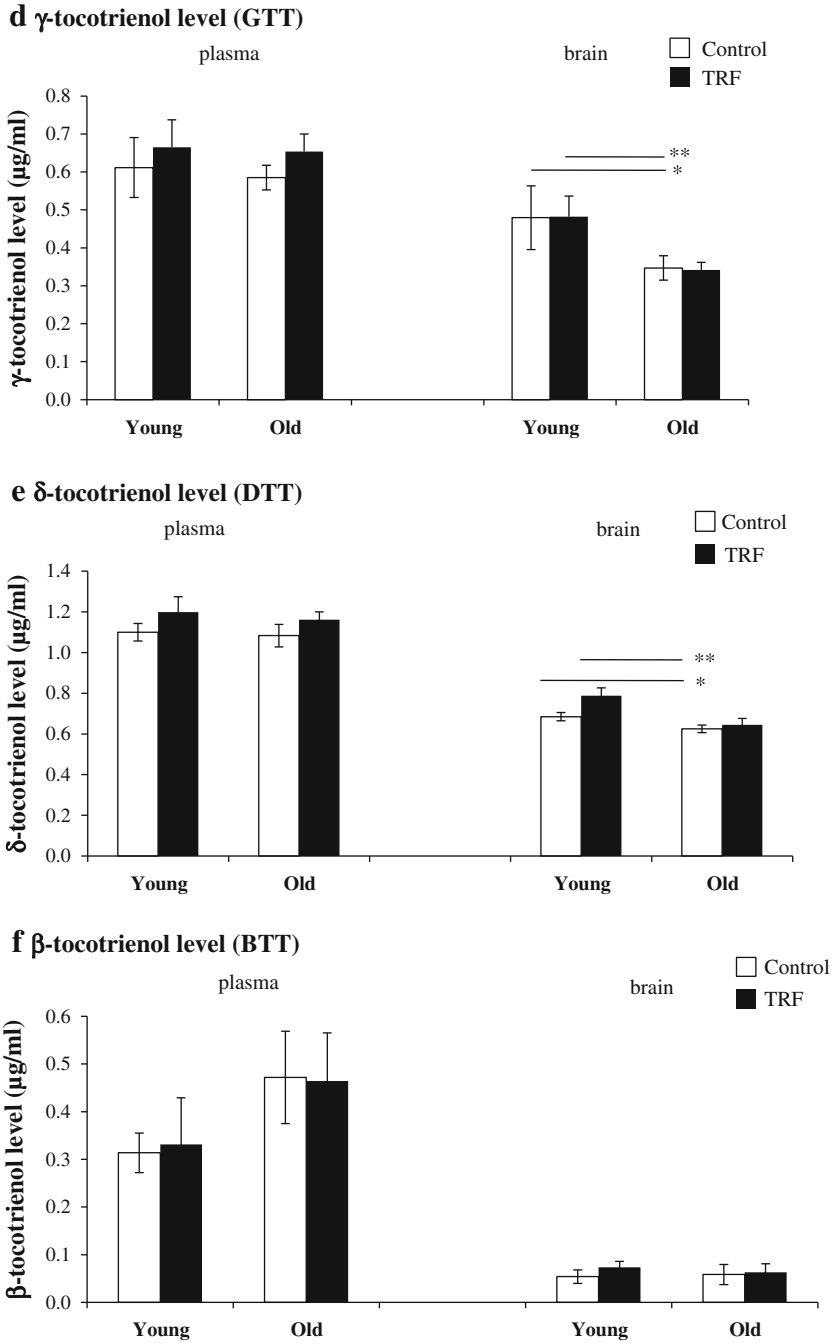


Fig. 9 Vitamin E level in plasma and brain tissue **a** total vitamin E, **b** α-tocopherol, **c** α-tocotrienol, **d** γ-tocotrienol and **e** δ-tocotrienol in young and old rats with and without tocotrienol rich fraction (TRF) supplementation. *A significant difference between young control vs. old control ($p < 0.05$). **A significant difference between young TRF vs. old control ($p < 0.05$). #A significant difference between old control vs. old TRF ($p < 0.05$)

Fig. 9 continued



Discussion

The potential benefits of nutrients to prevent or treat age-related behavioral impairments are currently a topic of interest, particularly in view of the increased aging population worldwide. Various studies have explored the health benefits of food-based or supplement-based nutrients, whether in the form of foods or supplements. Vitamin E has received considerable attention because of its antioxidant properties and its role in signal transduction pathways [26, 27].

Findings from the present study corroborate those of previous reports that aging is associated with behavioral impairments [3, 28]. Only the open field tasks were carried out in the current study, to determine exploratory, anxiety-related behavior and locomotor activity based on the natural tendency of rodents to explore novel environments [29]. A higher anxiety level is indicated by increased excretion of fecal boli, fewer grids crossed, and less wall-supported rearing. These responses were observed in aged rats when compared with their younger counterparts in the present study, indicating that aged rats were more anxious.

Further more, we found that aged rats exhibited reduced exploratory activity, shown by the lower number of grids crossed and less time spent in the center zone.

The MWM task is a hippocampus-dependent spatial learning test in which rats are required to learn the location of a hidden platform in the pool. This task incorporates both reference and working memory. Reference memory involves the ability to hold information for a period of time and working memory involves the ability to remember previously learned information for a relatively brief period [30, 31]. Over the 5 days of training in the present study, aged rats were found to take a longer time and path to find the hidden platform in the place navigation task, which tests for reference memory. Further more, the swim speeds of aged rats did not differ from the other groups. Overall, these results indicate age-related spatial learning deficits, which corroborate findings from previous studies [3, 28].

To determine if spatial memory was used to locate the hidden platform, rats were subjected to a probe test after the place navigation tasks. In the probe test, the platform was removed and the rats were allowed to search for it for up to 60 s. The percentage of time spent in the target quadrant and the numbers of platform crossings were assessed. A higher percentage of time spent in the target quadrant and the number of platform crossings were used as an index of memory retention [32]. Compared with young rats, aged rats spent less time in the target quadrant, suggesting the inability to remember which quadrant contained the platform. Further more, aged rats exhibited fewer platform crossings compared with their younger counterparts, and thus did not accurately locate the platform. These results suggest that aged rats have poorer spatial memory as compared with young rats, which is consistent with a previous study [3]. Aging may also affect perceptual-motor processing ability, thus possibly influencing these results. However, our findings revealed no significant difference in latency between all groups in the cued navigation task, thereby eliminating this possibility.

In the working memory test, the distance to reach the hidden platform was longer in aged rats compared with young rats at 1 and 60 min delays of the test phase. This finding indicates that working memory of the aged rats was markedly impaired compared with their young counterparts. Overall, the behavioral data from the present study confirmed earlier published reports that demonstrate spatial learning and memory deficits in aging animals [3, 33].

There are numerous theories to explain the age-induced behavioral impairment. One of these theories is the gradual accumulation of ROS, leading to damage [34]. Therefore, intake of antioxidants may prevent or reverse the detrimental effects of ROS. We have previously shown that long-term (8 months) supplementation of TRF to young rats prevents cognitive decline [12]. In the current study, we evaluated the effect of supplementing TRF to old rats to determine whether TRF can reverse the age-induced cognitive impairment.

Results from the open field test showed that TRF supplementation to the aged rats reduced the excretion of fecal boli and decreased rearing in addition to an elevated number of grid crossings and time spent in the center zone. These data indicate that TRF supplementation to aged rats reduces their anxiety level and increases their exploratory activity.

The place navigation test of the MWM task showed that aged rats supplemented with TRF displayed a faster time and shorter distance traveled to the hidden platform, indicating an improvement in spatial learning ability.

In the probe test, the percentage of time spent in the target quadrant of aged rats supplemented with TRF was not significantly different from that of age-matched controls. However, the number of platform crossings was significantly increased in aged rats with TRF supplementation.

In the working memory test of the MWM task, the path length 60 min after the sample phase was significantly improved in TRF supplemented aged rats compared with their age-matched controls. Overall, our findings indicate that TRF supplementation was able to reverse age-related behavioral impairments. Interestingly, TRF supplementation did not significantly affect the behavioral pattern of young rats. This response may be due to the intact cognitive function of these rats.

Our findings showed that aging increased the percentage of DNA and the severity of DNA damage. Lipid peroxidation also increased in aged rats, as shown by the elevated levels of plasma MDA. Aged rats displayed a decrease in SOD and an increase in CAT. However, GPx levels remained unchanged. TRF supplementation in aged rats decreased DNA damage and MDA levels. This treatment also increased the activity of SOD, CAT and GPx. These results corroborate previous findings that TRF supplementation reduces oxidative damage and improves the levels of antioxidant enzymes [12, 35].

Similar to the behavioral assessments, the effects of TRF supplementation were only observed in aged animals. This finding indicates that the therapeutic effect of TRF is effective under conditions of increased oxidative stress, which occurs during aging. We have also previously shown that TRF treatment improves wound healing in diabetic rats, which exhibit oxidative stress, but not in normal rats [37].

Results from the present study showed that total vitamin E levels in the plasma and brain decreased in aged animals. We have previously reported that a decrease in total vitamin E in brains of 11-month-old rats [38]. This decrease may be due to a decrease in intestinal absorption, transport

and/or uptake by cells. We showed that TRF supplementation increased plasma levels of total vitamin E, α-tocopherol and α-tocotrienol. Therefore, these isomers of vitamin E may be responsible for the protection against DNA damage and lipid peroxidation observed.

Our findings also revealed that the levels of vitamin E and α-tocotrienol increased in the brains of aged rats supplemented with TRF. Interestingly, although γ-tocotrienol is the predominant constituent in the TRF mixture, α-tocotrienol was found to be absorbed more readily. α-Tocotrienol is reported to be the most potent neuroprotective isomer of vitamin E [22] and may exert its neuroprotective effect by inhibiting glutamate-induced activation of phospholipase A2 [39]. α-Tocotrienol has also been reported to modulate the metabolism of arachidonic acid, which is one of the most abundant polyunsaturated fatty acids in the central nervous system [40].

Vitamin E is a powerful antioxidant and its isomers may also prevent neurodegeneration by modulating the apoptotic and survival pathways [10]. In the present study, the effectiveness of vitamin E in the aged rats may indicate an accumulation of ROS during aging. Free radicals may not only accumulate due to the productions of cellular metabolism, but may also be due to decrease levels of antioxidants with age because of poorer absorption, or intake of food. Epidemiological studies have shown that the elderly have higher rates of nutritional deficiencies, which include vitamin E [41, 42], and that low circulating levels of vitamin E isomers increases the risk of developing cognitive impairment and AD [43].

Conclusion

Our results suggest that TRF supplementation reverses the age-related deficits in spatial learning and memory and is associated with improved circulating oxidative status.

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Appendix

See Figs. 10, 11, 12, 13, 14.

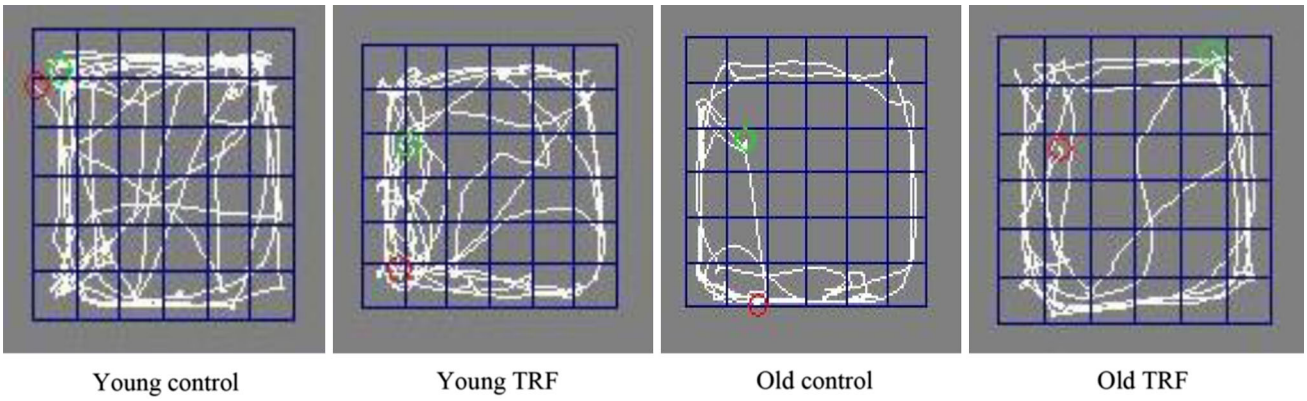

Fig. 10 Tracing diagrams of representative *squares* traversed by the rats in open field

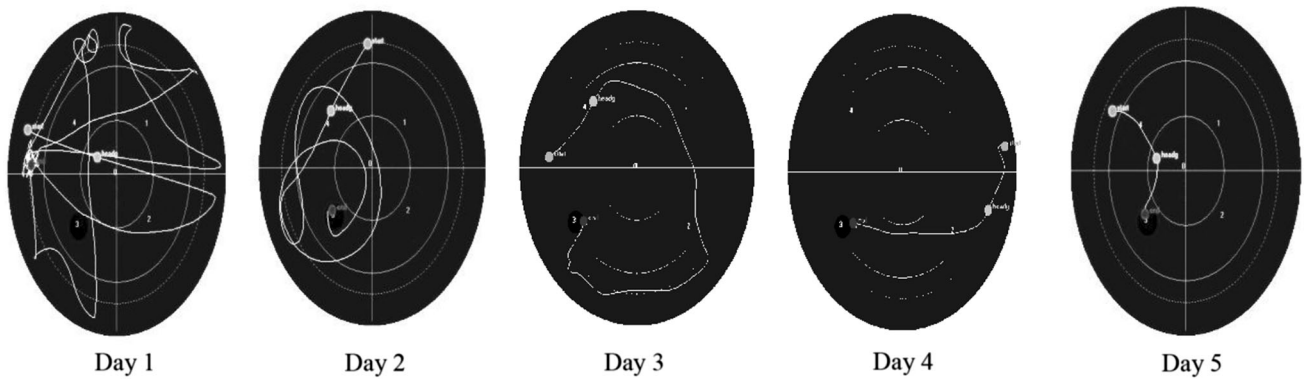

Fig. 11 Tracing diagrams of representative swim traces of the rats in navigation tasks of the Morris water maze test

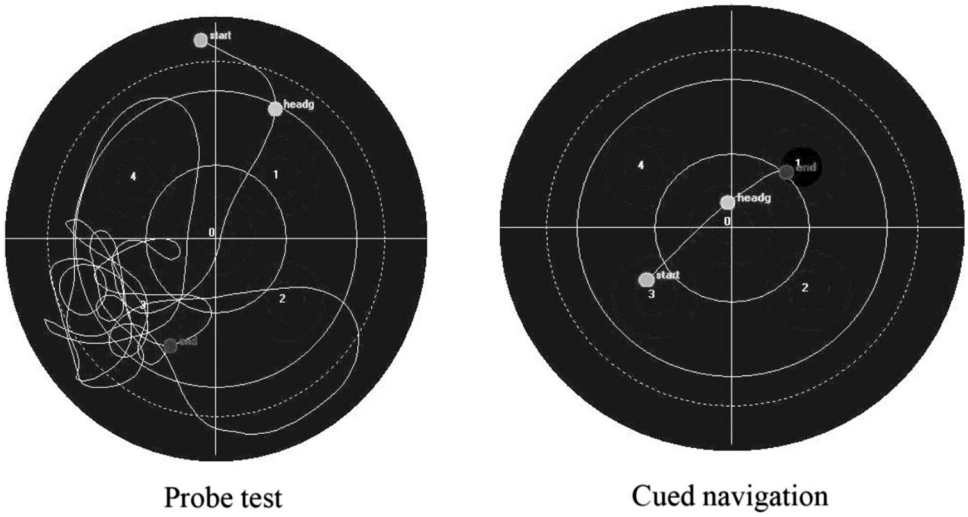

Fig. 12 Tracing diagrams in probe test and cued navigation tests

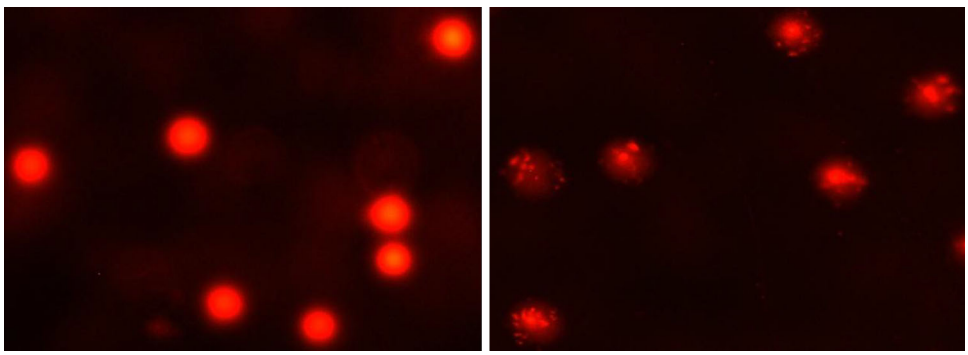

Fig. 13 Photograph of cells during comet assay analysis

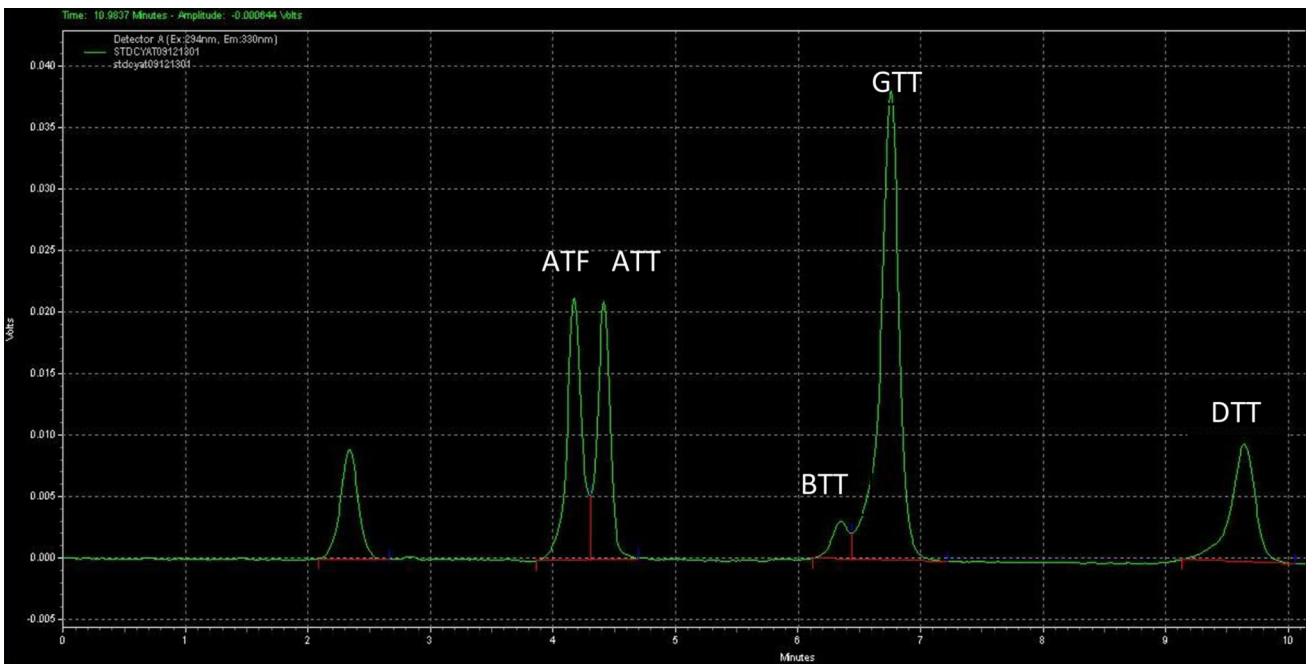

Fig. 14 Chromatogram of vitamin E isomers

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