



## Original article

# What is the combined effect of intense intermittent exercise and Ginkgo biloba plant on the brain neurotrophic factors levels, and learning and memory in young rats?



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## ARTICLE INFO

## Article history:

Received 26 August 2018

Received in revised form 6 January 2019

Accepted 11 February 2019

Available online 12 February 2019

## Keywords:

Brain-derived neurotrophic factor

Neurotrophin-4

Ginkgo biloba

Exercise

Hippocampus

Rat

## ABSTRACT

**Background:** The present study was conducted to investigate the effect of intense intermittent exercise and Ginkgo biloba on the hippocampal levels of brain-derived neurotrophic factor (BDNF) and neurotrophin-4 (NT-4) and also memory and learning in young rats.

**Methods:** Forty two eight-week-old rats were randomly divided into six groups including control, low dose of Ginkgo biloba (65 mg/kg), high dose of Ginkgo biloba (100 mg/kg), exercise, exercise + low dose of Ginkgo biloba, exercise + high dose of Ginkgo biloba. The exercise protocol or Ginkgo biloba administration was six days a week for six weeks. The hippocampal levels of BDNF and NT-4 were measured by ELISA method, and learning and memory were evaluated by Morris water maze test in all groups. Data were analyzed using SPSS software.

**Results:** Increase in hippocampal levels of BDNF and NT-4 appeared following exercise ( $p < 0.01$ ). The levels do not change following exercise + Ginkgo biloba administration. However, the NT-4 level decreased in the high dose of Ginkgo biloba group ( $p < 0.01$ ). Disorder in learning and memory was indicated following the use of low dose of Ginkgo biloba or exercise + low dose Ginkgo biloba administration ( $p < 0.001$ ). Learning elevated in the exercise group ( $p < 0.05$ ).

**Conclusions:** Exercise in young rats may increase brain neurotrophin levels and lead to improved learning. The preventative or protective role of Ginkgo biloba against some diseases has been suggested, but its consumption in young athletes is recommended with caution.

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## Introduction

Neurotrophins, a group of secreted proteins, are produced by structures in the nervous system [1]. The main member of the neurotrophins family, BDNF, is made by neurons, especially hippocampus and cortex neurons involved in learning and memory [2]. BDNF is a 14-kDa molecular weight neurotrophin that plays an important role in the development of the nervous system, synaptic activity, and improvement of cognitive function due to enhancing antioxidant enzymes, and anti-apoptotic agents

and structures involved in synapses by binding to tropomyosin receptor kinase B (TrkB) receptor [1]. The increase in BDNF associated with memory recovery has been shown in many studies [3,4].

Neurotrophin-4 (NT-4), the fourth member of the neurotrophin family, is one of the growth factors essential for neuronal growth, development and survival. This neurotrophin also maintains the integrity of the function and structure of the nervous system, and mediates the nerve plasticity in response to injury or disease [5]. The NT-4 acts via the TrkB receptor like BDNF. Since NT-4 has affinity with TrkB, its function is similar to BDNF [6].

Participating in sport activities and aerobic fitness is effective on better behavioral and cognitive performance of children, and young and old people. Recent research using MRI has shown that

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exercise can lessen the atrophy of brain tissue with aging [2]. It seems that exercise has a preventive role in different neurological diseases by increasing the content of brain neurotrophins [7].

Various studies have shown that some plants compounds enhance memory. The Ginkgo biloba supplement is one of the oldest of Asian plants with many properties. The most common application of this plant is to increase memory [8].

The consumption of Ginkgo biloba in people with mild motor disorder has increased serum BDNF and alleviated the symptoms [9]. Using this plant in young and old female rats did not affect serum BDNF levels, but improved cognitive function with reduced oxidative damage to the brain tissue [10]. The effect of Ginkgo biloba on NT-4 levels has not been reported so far.

On the one hand, given that the effect of ginkgo biloba on learning and memory and brain neurotrophins levels is unknown in young subjects in spite of aged subjects. On the other hand, exercise has been shown to boost memory, and change brain neurotrophins. Research on the effects of ginkgo biloba and exercise has been limited, despite the benefits of this plant on brain function.

Therefore, in this study, we tried to evaluate the combined effect of exercise and consumption of Ginkgo biloba in addition to the effect of single application of Ginkgo biloba or exercise on learning and memory and the brain levels of neurotrophins in young rats. To this end, learning and memory, as well as brain BDNF and NT-4 levels after six weeks of exercise, consumption of Ginkgo biloba or exercise with Ginkgo biloba in adult male rats were investigated. It is hoped that such a study would be useful in managing the consumption of this plant.

## Materials and methods

### Study animals

In this study, 42 male Wistar rats weighing 210–250 g (8–10 weeks age) were used. The animals were kept at the animal house of

the Kerman School of Medicine at a temperature of 20–22 °C and a 12-h dark/light cycle. The water and food were freely available to rats. The protocol was approved by the Ethics Committee of Kerman University of Medical Sciences (ID: IR.KMU.REC.1396.1168).

### Study groups

Male rats were divided into seven groups (n=6 in each group): control – healthy animals did not receive any substances, but their maintenance and feeding conditions were similar to other groups; vehicle – animals received the volume of Ginkgo biloba vehicle (water-alcohol-sorbitol solution), the same Ginkgo biloba volume; low dose of Ginkgo biloba – animals received 65 mg/kg of Ginkgo biloba [11]; high dose of Ginkgo biloba: animals received 100 mg/kg of Ginkgo biloba [12]; intense intermittent exercise – animals had intense intermittent exercise; intense intermittent exercise + low dose of Ginkgo biloba – animals had intense intermittent exercise and received 65 mg/kg of Ginkgo biloba; intense intermittent exercise + high dose of Ginkgo biloba – animals had intense intermittent exercise and received 100 mg/kg of Ginkgo biloba. Since there is no difference in variables between control group and vehicle group the results of vehicle group are not reported.

### Preparation of Ginkgo biloba

In this study, 65 mg/kg and 100 mg/kg of 6% solution of Ginkgo biloba powdered and dried leaves (Adonis Gol Dara, Tehran) were used. The vehicle of this plant powder contained a mixture of distilled water, sorbitol and alcohol, with ratios of 80, 5 and 15 respectively [11]. The vehicle or Ginkgo biloba solution was administered every morning by gavage six days a week for six weeks.

### Exercise protocol

After familiarizing and training the animals to run on a treadmill (5 days, 10 min a day at a speed of 10 m/min) [13],

**Table 1**  
Intense intermittent training protocol.

Week	Day	Odd day	Even day
Week 1	1	2 intervals, 40 m/min, 3 min	3 intervals, 54 m/min, 30 s
	2		
	3	2 intervals, 40 m/min, 3 min	5 intervals, 54 m/min, 30 s
	4		
	5	2 intervals, 40 m/min, 3 min	7 intervals, 54 m/min, 30 s
	6		
Week 2	1	3 intervals, 40 m/min, 3 min	9 intervals, 54 m/min, 30 s
	2		
	3	3 intervals, 40 m/min, 3 min	11 intervals, 54 m/min, 30 s
	4		
	5	3 intervals, 40 m/min, 3 min	13 intervals, 54 m/min, 30 s
	6		
Week 3	1	4 intervals, 40 m/min, 3 min	15 intervals, 54 m/min, 30 s
	2		
	3	4 intervals, 40 m/min, 3 min	17 intervals, 54 m/min, 30 s
	4		
	5	5 intervals, 40 m/min, 3 min	19 intervals, 54 m/min, 30 s
	6		
Week 4	1	5 intervals, 40 m/min, 3 min	19 intervals, 54 m/min, 30 s
	2		
	3	6 intervals, 40 m/min, 3 min	20 intervals, 54 m/min, 30 s
	4		
	5	6 intervals, 40 m/min, 3 min	
	6		
Week 5-6	1-12	6 intervals, 40 m/min, 3 min, to end of 6th week	20 intervals, 54 m/min, 30 s, to end of 6th week

intense intermittent exercise was performed six days a week for six weeks, with the principle of overload [6]. An intermittent exercise on even days included three minutes of warm up at a speed of 16 m/min followed by three minutes of intermittent running at a speed of 40 m/min. These intensities correspond to estimated energy outputs of 9.0 and 6.5 mL O<sub>2</sub>·100 g<sup>-1</sup> min<sup>-1</sup> or 95 and 68% VO<sub>2</sub>max. Finally, three minutes of cooling at a speed of 16 m/min was in plan. The active rest between each periodicity was 60 s at a speed of 16 m/min. The training program was initially two repetitions during the first week and increased to six repetitions until the fourth week. This protocol was maintained until the sixth week. Intermittent exercise on odd days included three minutes of warm up at a speed of 16 m/min, 30 s of running at a speed of 54 m/min. These intensities correspond to estimated energy expenditures of 9.5 and 6.5 mL O<sub>2</sub>·100 g<sup>-1</sup> min<sup>-1</sup> for rats or 100 and 68% VO<sub>2</sub>max. Finally, three minutes of cooling at a speed of 16 m/min was in plan. The active rest between each periodicity was 60 s at a speed of 16 m/min. The training program was initially three repetitions and increased to 20 repetitions until the fourth week. Then the same procedure was maintained until the sixth week (Table 1) [6].

#### Assessment of memory and learning

Memory and learning evaluation was performed using Morris water maze. The maze was a cylindrical pond with a diameter of 150 cm and a height of 60 cm filled with water to a height of 25 cm and temperature of 20°C. Black plexiglas platform with a diameter of 10 cm was 1.5 cm below the water level in the center of one of the four quadrants of the pond. The learning test was performed for three days as a block of four trials per day. To start the experiment, the rat was thrown from one of the four points starting within the pond and the animal was allowed 60 s to find the platform. If the animal was able to find the platform during this time, it was allowed to stay on the platform for 20 s. However, if the animal could not find the platform within 60 s, it was transferred to the platform. After completion of 20 s on the platform, the rat got out of the pond and then left the second starting point. This was done until the end of the fourth trial. After the fourth trial, the rat was warmed gently with a dry towel and then was restored to its cage. In each trial, the motion of the rat was filmed by a camera located above the pond and processed by the tracer software, and then information such as average elapsed time to reach the platform and average distance traveled to reach platform was obtained. Probe or memory test was performed on day four; the animal was released in a pond without the previous platform. Information such as time spent in the target quadrant and frequency arrival in the target quadrant that was previously a platform was extracted [14].

#### Measurement of hippocampus BDNF and NT-4 levels

After completing cognitive tests, the animals were anesthetized by intraperitoneal administration of ketamine (50 mg/kg) and xylazine (10 mg/kg). After brain removal, washing the hippocampus in brain was performed with PBS. Hippocampus samples were quickly frozen and stored in a freezer for collecting all samples.

After collecting samples, phosphate-buffered saline (PBS) solution containing ethylene diamine tetra acetic acid (EDTA) was prepared (160 g/Li) and added to hippocampal tissue (1:10; weight of the hippocampus: PBS solution containing EDTA) and then the mixture was homogenized in a cold environment and the supernatant was prepared by centrifugation.

The protein content of the supernatant was estimated using BCA protein assay reagent kit (Pierce) to ensure that an equal amount of protein from each sample was used. Supernatant

neurotrophins were measured by BDNF (sensitivity range 0.05–10 ng/mL) (CK-E30666, Eastbiopharm, China) and NT-4 (sensitivity range 15–4000 ng/mL) (CK-E30641, Eastbiopharm, China) ELISA kits, according to the instructions of kit manufacturer. Briefly, a quantitative assay of neurotrophins was done using the secondary antibody labeled with peroxidase and producing colored product to read in a spectrophotometer. A standard curve was prepared for any neurotrophin using respective standards, and the supernatant levels of neurotrophins were determined using the respective standard curve. The levels of BDNF and NT-4 were assessed in duplicate and expressed as picograms per milligram (pg/mg) and nanograms per milligram (ng/mg) of protein, respectively. Intra- and inter-assay precision tests were also done (CV < 10%, CV < 12%; respectively).

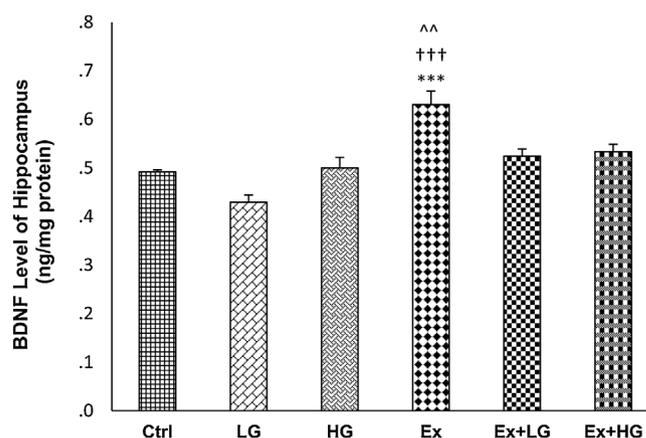
#### Statistical analysis

The collected data were analyzed using SPSS software version 20. The normality of data was examined using the Shapiro test. One way ANOVA was used to compare the mean of data in different groups followed by Tukey test, because of data normality. All data were expressed as mean ± SEM. The results of statistical analysis were considered significant with  $p < 0.05$ .

## Results

#### The combined effect of Ginkgo biloba administration and intense intermittent exercise on BDNF level in hippocampus

The level of hippocampus BDNF in study groups is shown in Fig. 1. In the present study, an increase in the level of hippocampus BDNF appeared after six weeks of intense intermittent exercise (0.63 ± 0.027 ng/mg of protein) compared to the control group (0.49 ± 0.004 ng/mg of protein) ( $p = 0.000$ ). There was no significant difference in the level of hippocampus BDNF among control, low dose of Ginkgo biloba and high dose of Ginkgo biloba groups. Also, the level did not change significantly after intermittent exercise + low dose of Ginkgo biloba and intermittent exercise + high dose of Ginkgo biloba (100 mg/kg). BDNF level in the intense intermittent exercise group was higher than other study groups (LG,  $p = 0.000$ ; HG,  $p = 0.000$ ; Ex+LG,  $p = 0.003$ ; Ex+HG,  $p = 0.009$ )



**Fig. 1.** The level of hippocampus BDNF in study groups (n = 6 in each group). Data was expressed as mean ± SEM. \*\*\*  $p < 0.001$  vs. Ctrl group; †††  $p < 0.001$  vs. HG group or LG group; ^^  $p < 0.01$  vs. Ex + LG group or Ex + HG group. Ctrl: Control; Ex: Intense intermittent exercise; LG: Low dose of ginkgo biloba; HG: High dose of ginkgo biloba; Ex + LG: Intense intermittent exercise + Low dose of ginkgo biloba; Ex + HG: Intense intermittent exercise + High dose of Ginkgo biloba.

### The combined effect of Ginkgo biloba administration and intense intermittent exercise on NT-4 level in hippocampus

The level of hippocampus NT-4 in study groups is shown in Fig. 2. The level of hippocampus NT-4 increased after six weeks of intense intermittent exercise ( $759.65 \pm 61.17$  ng/mg of protein) compared to the control group ( $439.46 \pm 25.82$  ng/mg of protein) ( $p = 0.000$ ). A decrease in the level was observed with high dose of Ginkgo biloba ( $212.30 \pm 32.12$  ng/mg of protein) in comparison to control group ( $p = 0.004$ ). This level in the intermittent exercise group was higher than the low dose of Ginkgo biloba ( $p = 0.038$ ), high dose of Ginkgo biloba ( $p = 0.000$ ), intermittent exercise + low dose of Ginkgo biloba ( $500.56 \pm 38.97$  ng/mg of protein,  $p = 0.001$ ) and intermittent exercise + high dose of Ginkgo biloba ( $449.78 \pm 17.19$  ng/mg of protein,  $p = 0.000$ ) groups. An increase in NT-4 level was observed in the intermittent exercise + low dose of Ginkgo biloba and intermittent exercise + high dose of Ginkgo biloba groups in comparison with low dose of Ginkgo biloba ( $p = 0.017$ ) and high dose of Ginkgo biloba groups ( $p = 0.000$ ).

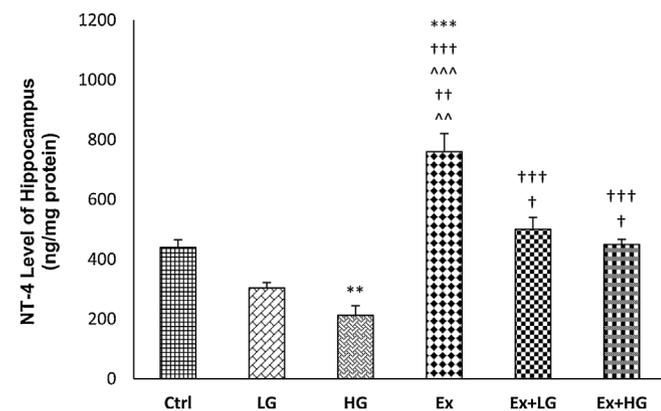
### The combined effect of Ginkgo biloba administration and intense intermittent exercise on learning

An increase in the mean of distance traveled to reach the platform was obtained in low dose of Ginkgo biloba compared to other groups ( $p = 0.000$ ). The amount of this mean in exercise + low dose of Ginkgo biloba group was higher than the control, intermittent exercise and exercise + high dose of Ginkgo biloba groups ( $p = 0.000$ ,  $p = 0.000$  and  $p = 0.015$ , respectively) (Fig. 3A).

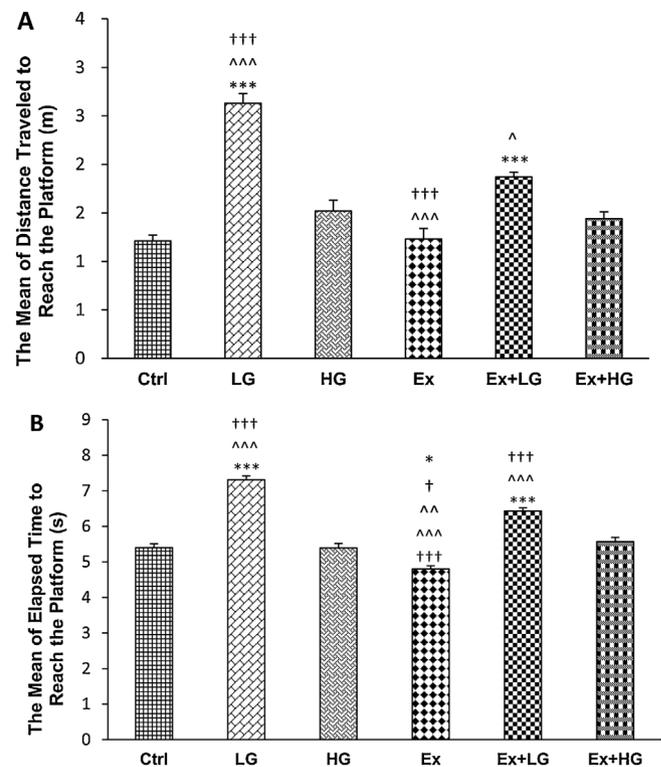
The mean of elapsed time to reach the platform in low dose of Ginkgo biloba and exercise + low dose of Ginkgo biloba groups was higher than other groups ( $p = 0.000$ ). This mean following intermittent exercise was reduced in comparison to control ( $p = 0.033$ ), high dose of Ginkgo biloba ( $p = 0.036$ ), and exercise + high dose of Ginkgo biloba ( $p = 0.003$ ) groups (Fig. 3B).

### The combined effect of Ginkgo biloba administration and intense intermittent exercise on memory

The results of time elapsed in the target quadrant in the memory test of study groups is shown in Fig. 4. The mean of time in exercise + low dose of Ginkgo biloba group was lower than other groups ( $p = 0.000$ ) (Fig. 3A).



**Fig. 2.** The level of hippocampus NT-4 in study groups ( $n = 6$  in each group). Data was expressed as mean  $\pm$  SEM. \*\*\*  $p < 0.001$ : vs. Ctrl; group;  $p < 0.001$ : vs. LG group or HG group; \*\*\*  $p < 0.001$ : vs. Ex + HG group; \*\*  $p < 0.01$  vs. Ex + LG group; \*\*  $p < 0.01$  vs. Ctrl group;  $p < 0.01$  vs. HG group;  $p < 0.05$  vs. LG group or HG group. Ctrl: Control; Ex: Intense intermittent exercise; LG: Low dose of ginkgo biloba; HG: High dose of ginkgo biloba; Ex+LG: Intense intermittent exercise + Low dose of ginkgo biloba; Ex + HG: Intense intermittent exercise + High dose of Ginkgo biloba.

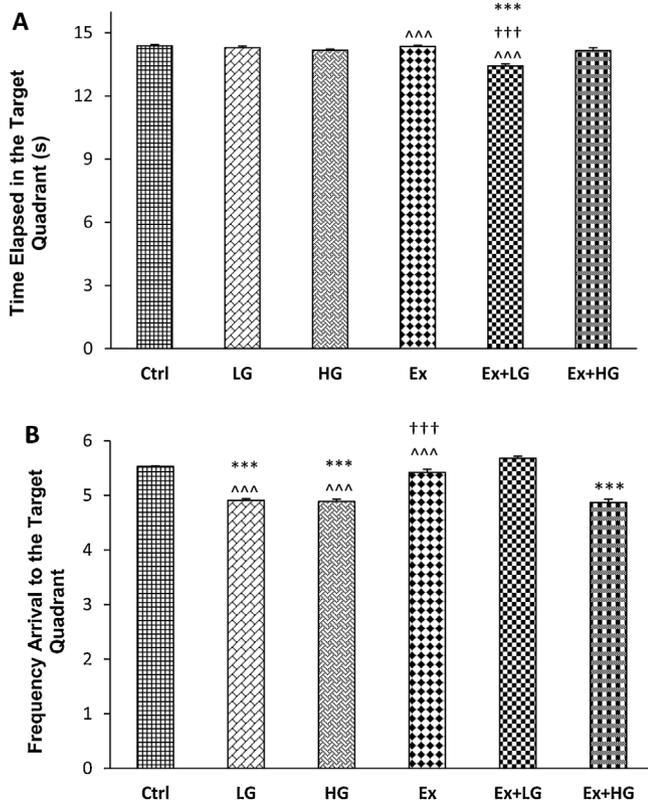


**Fig. 3.** The combined effect of intense intermittent exercise and administration of Ginkgo biloba on learning test ( $n = 6$  in each group). Data was expressed as mean  $\pm$  SEM. A: The mean of distance traveled to reach the platform in study groups. \*\*\*  $p < 0.001$  vs. Ctrl group; \*\*\*  $p < 0.001$  vs. HG group or LG group; \*\*\*  $p < 0.001$  vs. Ex + LG group or Ex + HG group; ^  $p < 0.05$  vs. Ex + HG group. B: The mean of elapsed time to reach the platform in different days of learning in study groups. \*\*\*  $p < 0.001$  vs. Ctrl group; \*\*\*  $p < 0.001$  vs. HG group or LG group; \*\*\*  $p < 0.001$  vs. Ex + LG group or Ex + HG group; ^  $p < 0.01$  vs. Ex + HG group; †  $p < 0.05$  vs. Ctrl group; †  $p < 0.05$  vs. HG group. Ctrl: Control; Ex: Intense intermittent exercise; LG: Low dose of ginkgo biloba; HG: High dose of ginkgo biloba; Ex + LG: Intense intermittent exercise + Low dose of ginkgo biloba; Ex + HG: Intense intermittent exercise + High dose of Ginkgo biloba.

The results of frequency arrival to the target quadrant in the probe test are shown in Fig. 4. The number of arrivals to the target quadrant was low in the low dose of Ginkgo biloba, high dose of Ginkgo biloba, and exercise + high dose of Ginkgo biloba groups in comparison to control group ( $p = 0.000$ ). In addition, a reduction in the number appeared in the low dose of Ginkgo biloba and high dose of Ginkgo biloba groups compared to exercise + low dose of Ginkgo biloba ( $p = 0.000$ ). In contrast, an increase in frequency was observed in exercise group in comparison to low dose of Ginkgo biloba, high dose of Ginkgo biloba, exercise + low dose of Ginkgo biloba ( $p = 0.000$ ) groups (Fig. 4B).

## Discussion

For the first time, the combined effect of intense intermittent exercise and Ginkgo biloba plant on learning and memory, and brain neurotrophic factors levels in young male rats was investigated in current study. The results of this study were 1) the hippocampus BDNF and NT-4 levels increased following intense intermittent exercise; 2) The combined application of intermittent exercise and the Ginkgo biloba did not affect hippocampus BDNF and NT-4 levels; 3) Learning disorder was observed following the use of low dose Ginkgo biloba and also combination of exercise and low dose Ginkgo biloba; 4) The application of Ginkgo biloba alone or in combination with exercise resulted in memory disturbance.



**Fig. 4.** The combined effect of intense intermittent exercise and administration of Ginkgo biloba on probe test ( $n=6$  in each group). Data was expressed as mean  $\pm$  SEM. A: Time elapsed in the target quadrant in study groups. \*\*\*  $p < 0.001$  vs. Ctrl group; †††  $p < 0.001$  vs. HG group or LG group; ^^^  $p < 0.001$  vs. Ex + LG group or Ex + HG group. B: Frequency arrival to the target quadrant in study groups. \*\*\*  $p < 0.001$  vs. Ctrl group; †††  $p < 0.001$  vs. HG group or LG group; ^^^  $p < 0.001$  vs. Ex + LG group or Ex + HG group. Ctrl: Control; Ex: Intense intermittent exercise; LG: Low dose of ginkgo biloba; HG: High dose of ginkgo biloba; Ex + LG: Intense intermittent exercise + Low dose of ginkgo biloba; Ex + HG: Intense intermittent exercise + High dose of Ginkgo biloba.

The important role of neurotrophins has been raised in relation to cognitive and learning disorders, and extensive research is performed to eliminate existing contradictions [15]. BDNF is a leading neurotrophin for learning, memory, and neuronal flexibility, and plays an important role in controlling neuronal disorders and genetic diseases such as Alzheimer's, Parkinson's, severe depression, epilepsy, etc. [16].

In the present study, the levels of hippocampus BDNF and NT-4 increased after intense intermittent exercise. In agreement with this result, it has been reported that prolonged voluntary exercise at middle-age causes neurogenesis of the hippocampus and an increase in the level of the hippocampus BDNF [17]. In line with our results, walking on the treadmill in situation of damaged peripheral nerves increased the rate of neurogenesis associated with enhancing NT-4 level in damaged nerve [18]. It has been shown that intense exercise has a preventive role against neurological diseases by increasing the level of brain neurotrophins [7]. Therefore, intense intermittent exercise may result in an increase in the level of neurotrophins involved in cognitive function. Contrary to these results, the change in the level of hippocampus BDNF has not been reported following exercise on the treadmill and also the running wheel in some studies [19].

Generally, an increase in the conversion of Pro-BDNF [20], IGF-1 [21], estrogen [22], levels of oxidative and inflammatory activity [7], production of NO [23], and reduced corticosterone concentration [22] have been proposed as potential mechanisms for increasing BDNF following exercise, which can change according to the intensity

of exercise and sex. In contrast, the regulation of the expression of myelin-dependent glycoproteins, myelin basic proteins, and low-molecular weight neurofilament proteins may be mechanisms responsible for NT-4 enhancement following exercise [24].

In the results of this study, although the increase in learning was observed in exercised animals, intense intermittent exercise did not affect memory. Similarly, Taati et al. showed exercise improves synaptic plasticity and spatial learning [25]. In contrast, spatial learning was not affected by running in another study [26]. In addition, the beneficial effect of physical activity on cognitive functions has not been observed in a human study [27]. It seems an increase in the gene expression of presynaptic proteins including synaptotagmine and synaptophysin in the pyramidal cells of hippocampus CA1 region can facilitate the release of stimulatory neurotransmitters in the synaptic space [28] resulting in the enhancement of synapse efficiency and learning.

It has been reported the exercise in some patterns affects memory only in elderly animals, not young animals [29]. In addition, the role of various factors such as exercise protocol (optional or compulsory), intensity of exercise and duration of exercise [30] has been suggested to affect learning and memory. Stress in exercise can affect memory and reduce it [31]. The cell proliferation in dentate gyrus of the rats is determined by the intensity and duration of the exercise [32]. Consequently, the contradiction of the results may be due to differences in duration, intensity, and protocol of exercise. The results of this part of the current study suggest that exercise with this pattern and severity does not affect memory in young healthy rats, and an increase in neurotrophins levels and learning is not associated with memory enhancement in these rats.

In the present study, although the level of hippocampus BDNF did not change following the administration of Ginkgo biloba, a memory amplifier plant, the level of hippocampus NT-4 reduced. Contrary to our finding, five-week treatment with Ginkgo biloba increased the expression of brain BDNF, as well as improved motor function in motor disorder [33]. Indeed, the Ginkgo biloba caused learning and memory impairment along with the decrease or the loss of change in the hippocampal neurotrophins level in the current study. However, it was found in a study that healthy men receiving Ginkgo biloba for five days had an increase in memory score [34]. The difference in results could be related to dose, time, method and duration of administration in addition to exercise factors, and subjects' age and situation. Therefore, the administration of Ginkgo biloba has to be considered with caution in healthy young subjects even in presence of exercise.

In current research, the combined application of Ginkgo biloba and exercise did not affect the level of brain NT-4 and BDNF, whereas it reduced learning and memory. It seems the exercise in combined application prevented a decrease in NT-4 by administration of Ginkgo biloba.

Consequently, it is supposed the Ginkgo biloba reduces learning and memory in young subjects through other mechanisms in addition to hippocampus NT-4 decrement. The latter requires research for confirmation. However, exercise probably augments learning in part by increasing hippocampus neurotrophins.

Therefore, exercise in young subjects may increase brain neurotrophins levels and lead to preventing Alzheimer in aging. Although the preventative or protective role of Ginkgo biloba against some cognitive diseases has been suggested, its consumption in young athletes is probably not effective on learning and memory. The limitation of this study was using alone young exercised rats. It is proposed doing this study in aging exercised rats.

## Conclusion

Exercise in young rats can increase brain neurotrophins level leading to augmentation of learning. Although Ginkgo biloba has

been proposed as a memory enhancer and is also preventative or protective against some diseases, it should be consumed with caution in the young subjects.

### Conflict of interest

The authors declare no conflict of interest.

### Author contributions

All authors have made substantial contributions to this work: ZS and MK supervised and directed the project, contributed to the data analyses and interpretations, and prepared the manuscript. MS: collected the data. MSA: directed the project, and carried out the interpretations. MP: carried out the interpretations and revised final version of manuscript. All authors read and approved the final manuscript.

### Acknowledgments

The authors would like to thank Mr. Sadeghinejad, Mr. Bejeshk, Mr. Nouri and Mr. Gohargazi for their collaboration in the execution of this study.

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