Title: The Effects of Ripe Pistachio Hulls Hydro – Alcoholic Extract and Aerobic Training on Learning and Memory in Streptozotocin – Induced Diabetic Male Rats

Running Title: The Ripe Pistachio Hulls and Aerobic- Training Effects on Learning & Memory

Authors: Sajad Abdi Gorabi¹, Hasan Mohammadzadeh ¹, Mohammad Rostampour ²*

¹ Department of Motor Behavior, Faculty of Physical Education and Sport Sciences, University Of Urmia, Urmia, Iran. Email: sajad.abdi2015@gmail.com

² Cellular and Molecular Research Center, Guilan University of Medical Sciences, Rasht, Iran; Department of Physiology, Guilan University of Medical Sciences, Rasht, Iran
   Email: rostampour@gums.ac.ir

* Cellular and Molecular Research Center, Guilan University of Medical Sciences, Rasht, Iran; Department of Physiology, Guilan University of Medical Sciences, Rasht, Iran. Mobile number: +9111309782; Phone number: +98 13 33690099; Fax: +98 13 33690036;
   Email: rostampour@gums.ac.ir; rost_v@yahoo.com

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ABSTRACT

Introduction: Diabetes mellitus has harmful effects on body functions such as learning and memory. According to the role of exercise and medicinal plants on body health, the purpose of this study was to survey the effect of combined aerobic training and use of ripe pistachio hulls (RPH) hydro-alcoholic extract on Learning and memory in streptozotocin – induced diabetic male rats.

Methods: In this experimental study, 42 male wistar rats weighing 250-280 g were used in 6 groups with an equal number of 7 rats in each one. Rats were diabetic with streptozotocin (STZ) (50 mg / kg), and the test protocol was applied for 8 weeks. Passive avoidance memory was assessed by using a step through passive avoidance apparatus (Shuttle Box). SPSS software was used to analyze the data and P < 0.05 was significant.

Results: The results showed that step-through latency in the acquisition trial (STLa) was not significantly different among groups. While step-through latency in 24 h retrieval (STLr 24) test was significantly reduced and time spent in the dark compartment (TDC) was decreased in treated groups compared to diabetic control groups (p < 0.001), there was no significant difference between the STZ and saline diabetic groups.

Discussion: The findings of this study revealed that the RPH hydro-alcoholic extract and aerobic exercise could improve passive avoidance memory in streptozotocin diabetic rats. Meanwhile, they might be an adjuvant therapy together with other traditional medicine.

Key words: Ripe pistachio hulls, Aerobic training, Learning and memory, Streptozotocin - diabetic rats
1. Introduction

Diabetes mellitus is one of the most serious endocrine disorders. It is estimated that over 347 million people worldwide have suffered from this disease until 2011 (Danaei et al., 2011). The World Health Organization (WHO) has predicted that the number of people with diabetes will increase to 366 million in 2025 (Young et al., 1992). Also, the WHO reported that nearly 35.6 million people live with dementia. It is expected that this number might be doubled by 2030 (65.7 million), and it can reach more than triple rate by 2050 (115.4 million) (Ammari et al., 2018). New ways of urban living, lack of exercise and unhealthy nutrition, along with the consumption of fatty and sweet foods, are among the factors that cause health damage, the development of metabolic diseases, and the loss of human life. One of the metabolic diseases is diabetes. Diabetes is an autoimmune disease that various genetic factors, environmental stress, viral infections, and diet can bring about the destruction of the insulin-producing beta cells in the pancreas. Disruption of cognitive and non-normalization of neurochemical compounds and brain structures in diabetic patients and rodent diabetic rats with streptozotocin (STZ) has been reported (Young et al., 1992; Ogonovszky et al., 2005). Furthermore, memory and learning, ability to solve problems, speed of exercise, mental and complex physical movements, inference and understanding of the person are damaged in diabetic patients (Ramanathan & Jaiswal, 1998).

Many studies have been conducted on the treatment of diabetes using medicinal herbs. The protective effects of plant extracts including: cumin, fenugreek, sage, moss, etc., have been confirmed in diabetic rats (Shahbazi & Maleknia, 2008). Pistachio is one of the most widely used herbs in traditional medicine. Hull is the largest by-product of pistachio (accounting for on average between 35% and 45%) during industrial post-harvest processing (Grace et al., 2016; Barreca et al., 2016). It was reported that ethanol extract of RPH contains large
diversity of phenolic acid, flavonoids, flavonols, flavones and flavonols (Barreca et al., 2016). Based on its constituents, RPH could be a cost-effective source of compounds with health protective potential. Also, RPH phenolic derivative might be used as a natural alternative to synthetic antioxidants (Grace et al., 2016; Sevcan, Ozlem, Reinhold, & Ralf, 2017). The role of flavonoids in memory enhancement has been shown (Nishimura, Taki & Takaishi, 2000).

There are reports that aerobic exercises could improve memory and learning (Khalaj & Ahmadi, 2016). Also, physical activity has a critical role in blood glucose regulation and health in pre-diabetic and diabetic persons (Colberg et al., 2016). Several studies have been shown that exercise both in human and animal models improve cognition and learning and memory (Yi, 2015). Considering the fact that exercise and some medicinal plants can have beneficial effects on memory in diabetic rats, the present study was designed to investigate the effect of ripe pistachio hulls (RPH) hydro-alcoholic extract and aerobic exercise on learning and memory of streptozotocin-diabetic male rats.

2. Methods

2.1. Chemicals and preparation of ripe pistachio hulls hydro-alcoholic extract

Streptozotocin was purchased from Sigma-Aldrich (St. Louis, MO, USA) and was dissolved in saline.

The ripe pistachio (Pistacia vera L.) nuts of Ohadi cultivar were obtained from 13 year-old trees grown in Damghan region of Semnan Province in Iran in 2013. The plants were authenticated by associate professor Davood Bakhshi, a pomologist in the Faculty of Agriculture, University of Guilan, Rasht, Iran. The fleshy fruit wall of fresh and ripe Pistachios was removed and dried in an oven at 40 °C. Fifty grams of fine powder of RPH
was mixed with 70% ethanol (70% ethanol and 30% distilled water) and placed on a shaker incubator with gentle shaking for 72 h. Afterwards, the liquid phase of the container was placed on the funnel and then dried. The dried extract was weighted and re-suspended in normal saline. Animals received an appropriate amount of extract according to their weight and protocol. Meanwhile, the administration of the extract in saline solution was carried out by the gavage method (10 ml/kg).

Various doses of RPH hydro-alcoholic extract (1, 5, 10, 50, 100 and 500 mg/kg) were administered to rats by gavage for 2 months before training, and only 10 mg/kg dose of the extract significantly increased the STLr 24 h compared to saline group (P < 0.05) and there was a significant reduction in the TDC compared to the sham and saline groups (respectively, P < 0.01 and P < 0.05). Therefore, 10 mg/kg dose of RPH extract was selected as an effective dose.

2.2. Animals

In this experimental study, 42 male wistar rats weighing 250-280g were used with equal number (n = 7) which were kept at the Animal house of Guilan University of Medical Sciences at a temperature of 20 ± 2 °C and a light-dark cycle of 12 hours (the start of lighting at 9 am and start of darkness at 9 pm) 4 rats per cage with standard water and food. All behavioral tests were conducted from 10 am to 4 pm. All animal experiments were carried out in accordance with the national institute of health guide for the care and use of laboratory animals’ publication No. 85-23 revised in 1985. All protocols were also approved by ethical and supervisory guidelines of working with experimental animals of Guilan University of Medical Sciences with code No. (1930349905). The cages were cleaned twice a day, and during this time they were checked for their sugars, and if they reached the fetal border, they
were injected with insulin. Rats were randomly divided into six groups of 7: (1) sham group, which was not diabetic and did not get any medicine or exercise (2) STZ group, which was diabetic, but did not get any medicine or exercise; (3) The diabetic group, in which rats were received normal saline as solvent of RPH (STZ + Saline); (4) The diabetic group in which rats were received RPH hydro-alcoholic extract (STZ + RPH); (5) The diabetic group in which rats were running on the treadmill for 8 weeks and were training after 24 hours (STZ + Exercise); (6) The diabetic group in which rats received RPH hydro-alcoholic extract plus aerobic exercises.

2.3. Induction of diabetes

After controlling and recording the weight, the intravenous blood glucose of all animals was measured by using aAccu Check and the fasting blood glucose level was less than 200 mg/dl without fasting. Then, after 24 hours of fasting, rats were diabetic with an intraperitoneal injection of STZ at a dose of 50 mg /kg (Firouzjaei, Jafari, Eskandari, Anarkoli, & Alipour, 2013). For injection, STZ was dissolved in cold saline (1 ml/kg) solution, injected at 8 o'clock in the morning and in fasting state to the rats. Three days (72 hours) after STZ injection, blood glucose was monitored in animals. The criterion for diabetic rats was considered to be above 250 mg/dl, and therefore, rats without a blood glucose level above 250 mg/dl were excluded from the study (Firouzjaei, Jafari, Eskandari, Anarkoli, & Alipour, 2013; Hasanein & Shahidi, 2012; Jabbarpour, Shahidi, Saidijam, Sarihi, Hassanzadeh, & Esmaili, 2014).

2.4. Aerobic exercises

At first, rats passed the introductory period by rat treadmill. During this period, recurring short-term training courses were used during four consecutive days (Each training session included a 5-minute course of 10 meters per minute and 1 time per day). The untrained
animals were eliminated after the introductory period. One day was restored to the animals and then the main phase of training began. In the first week, animals ran a treadmill with a gradient of 0 and a speed of 10 m/min for 10 minutes. In the second week after 5 minutes running at speed of 10 m/min, speed reached to 20 m/min with the same zero gradient and running for 10 minutes. This period of practice continued for 8 weeks (Verma & Bordia, 1998). After a period of practice, animals of different groups were tested using standard devices for learning and memory.

2.5. Passive avoidance learning and memory

After a period of 8 weeks practice, the passive avoidance learning and memory of different diabetic groups of animals were evaluated using Shuttle Box apparatus which was purchased from Borj sanat company. The learning and memory measurement method in this study was Passive avoidance Learning. Long-term memory evaluation is based on negative reinforcement. This instrument had two parts of the compartment, the mechanical part and the controller, or the electronic part. Two chambers were separated by an 8 x 8 cm guillotine door. The dimensions of each chamber were 20 × 21 × 20 cm. On the floor of each of these two chambers, the bars of 2.5 mm in diameter are spaced apart with 1cm which acts as a network for transporting electric shock to an animal. The control unit determines the duration, frequency and shock intensity. The Habituation stage consists of two steps that take place at intervals of 30 minutes. At this stage, the rat was placed in a white chamber and the guillotine door was opened in 10 seconds and step-through latency was recorded until the animal entered the dark chamber. When animal entered to the dark chamber, the guillotine door was closed and the animal was taken out of the dark chamber, and after each stage the animal is placed in its individual cage. The training took place 30 minutes after the second stage of getting used to. Thus, the rat was placed in the white side, and after 10 seconds the
guillotine door was raised, The time of arrival of the animal was recorded in the acquisition latency, and the animal was closed at the entrance and the electric current was fed to the animal's legs for 1 second at a frequency of 50 Hz for 2 seconds. After 120 s this work was repeated. When the rat did not enter the dark chamber, the test was stopped; But again, the shock came when the animal entered the dark chamber. After three tests, if the mouse was not able to complete the task, it was removed from the test. This step was performed 24 hours after training to assess long-term memory. The test lasted for 10 minutes. Two timers were used to record the desired time for the test. At first, the rat was placed in a white chamber and the guillotine door was opened after 10 seconds, and step-through latency of animal into the dark chamber was recorded. Finally, the total time spent in the dark chamber was recorded for 10 minutes (Firouzjaei, Iafari, Eskandari, Anarkoli, & Alipour, 2013).

2.6. Statistical analysis

Data were analyzed using SPSS statistical program. Information was shown as mean ± standard error. The Kolmogrov-smirnov test was used to ensure the normal distribution of data in all measurement factors. If the distribution was normal, the results were compared using the one-way ANOVA test, followed by post-hoc Tuckey’s test. In the case of abnormal data, the Kruskal-Wallis statistical test was used. P <0.05 was considered as a significant level.

3. Results

3.1. The amount of step through latency in acquisition trial (STLa)

The results showed that the STLa increased in sham, STZ + RPH, STZ+ exercise and STZ+ RPH + Exercise groups in comparison with STZ and STZ + Saline groups, but these increases were not significant (Fig.1).
Fig 1. Comparison of STLa in different groups of Sham, STZ, STZ + Saline, STZ + RPH, STZ + Exercise and STZ + RPH + Exercise in passive avoidance learning test, (Mean ± SEM, n = 7).
3.2. Step through latency in 24 h retrieval (STLr24h)

24-hour post-training memory test showed that there was a significant difference between the lag time of the animal from the light chamber to the dark one at the reminder stage in comparison with the groups (p < 0.001). The intra-group comparison showed no significant difference between STZ and saline diabetic groups. On the other hand, there were significant differences in STZ and saline diabetic groups compared to sham, STZ + RPH, STZ+ exercise and STZ+ RPH + Exercise groups (p < 0.001) (Fig. 2).

![Graph showing comparison of STLr24h in different groups](image)

Fig 2. Comparison of STLr24h in different groups of sham, STZ, STZ + saline, STZ + RPH, STZ + Exercise and STZ + RPH + Exercise in passive avoidance learning test, *** P < 0.001, Compared to STZ and STZ + saline diabetic groups (Mean ± SEM, n= 7).

3.3. Time spent in the dark Compartment (TDC)
Intra-group comparisons showed no significant difference between STZ and saline diabetic groups. On the other hand, there were significant differences in STZ and saline diabetic groups compared to sham, STZ + RPH, STZ + exercise and STZ + RPH + exercise groups (p < 0.001) (Fig. 3).

![Fig 3. Comparison of TDC in different groups of sham, STZ, STZ + saline, STZ + RPH, STZ + Exercise and STZ + RPH + Exercise in passive avoidance learning test, *** P <0.001, Compared to STZ and STZ + saline diabetic groups (Mean ± SEM, n= 7).](image)

4. Discussion

In the present study, the effect of RPH hydro-alcoholic extract and aerobic training on learning and memory in STZ – induced diabetic male rats was studied. The results of this study showed that each of aerobic exercise and RPH hydro – alcoholic extract alone or together with each other improved learning and memory deficits in diabetic rats that the memory improvement in treated groups was significant compared to diabetic control groups.
STZ-induced diabetes reduces memory performance (Flores-Gómez, de Jesús Gomez-Villalobos, & Flores, 2018). In previous studies, learning and memory deficits have been reported in both diabetic human and animals (Patil, Singh, & Kulkarni, 2006; Kucukatay, Agar, Gumuslu, & Yargicoglu, 2007). In the memory test in diabetic rats, the latency in the dark chamber of the device (STLr) decreased and the time spent in the dark chamber (TDC) increased, indicating memory impairment following diabetes mellitus. The increase of step through Latency to the dark chamber cannot be attributed to the sensory or motor impairment of the animals (Oliyani, Balouchi, & Azadi, 2010). Because the amount of (STLa) before the electric shock did not show a significant difference among the groups.

In the present study, the shuttle box was used to measure passive avoidance memory. Feeding the hydro-alcoholic extract of RPH at a dose of 10 mg / kg caused a significant increase in STLr 24 h to the dark chamber and a significant reduction in the TDC compared to diabetic control groups. These results indicated that RPH extract improves memory impairment due to diabetes mellitus in rats. Phenolic compounds were found in the pistachio, with antioxidant, anti-inflammatory and antimicrobial effects (Azadedel, Hanachi, & Saboora, 2017). In epidemiological studies, it was proven that phenolic flavonoids, as antioxidants in pistachio red peal, have improved memory performance in older rats (Mozdastan, Ebrahimzadeh, & Khalili, 2015).

Pistachio red skin has a parenchymal and fibrous structure. It also consists of minerals such as potassium, magnesium, calcium, sodium. Meanwhile, flavonoids are important compounds in this plant, which could increase cholinergic activity (Zhang, Tang, Zheng, Chen, & Shen, 2011; Gao, Tang, He, & Bi, 2012) and learning and memory (Thirugnanasambantham, Viswanathan, Mythirayee, Krishnamurty, Ramachandran, & Kameswaran. 1990; Savelev, Okello, & Perry, 2004). It was also proven that acetylcholine receptor stimulation increases
memory and cognition (Baxter & Crimins, 2018), and it seems RPH extract increased memory through potentiation of cholinergic system.

The consumption of foods rich in polyphenols, including flavonoids, has a major impact on preserving brain function, improving learning and memory ability, and also contributes to the improvement of neuronal signaling in memory formation. Flavonoids have potent antioxidant and anti-inflammatory effects, which slow down the process of reducing brain function due to age. Flavonoids can improve blood flow to the brain and play an important role in increasing memory and learning ability. Possible mechanisms of the effects of flavonoids on brain function are effective processes for cell survival, differentiation and memory formation. The mechanism of action of these compounds is different in the brain. Some act as estrogens, and a group through neural signaling is involved in controlling morphological mechanisms that affect memory formation, thereby enabling effective enzymes in these pathways to increase memory and learning ability (Laurin, Verreault, Lindsay, MacPherson, & Rockwood, 2001; Van, Kempermann, & Gage, 1999). Some evidences have revealed that flavonoid-rich plants and foods could improve memory and synaptic plasticity. Flavonoids play an important role in improving the human memory and neurocognitive performances by protection of vulnerable neurons, and in elevate existing neuronal function, and neuronal regeneration (Spencer, 2008; Viggiano et al., 2006; Wang, Wang, Wu, & Cai, 2006).

In this study, exercise can improve the passive avoidance memory deficiency due to diabetes. Also, RPH consumption potentiated the incremental effect of exercise on memory. Various studies have confirmed the beneficial effects of physical activity and exercise on brain functions such as memory enhancement (Grealy, Johnson, & Rushton, 1999), cognitive function (Johansson & Ohlsson, 1996), neurogenesis (Ohlsson & Johansson, 1995), and improvement of brain injuries (Ogonovszky et al., 2005). Exercise, without adverse effects,
prevents repression of cell proliferation due to STZ in the sinusoidal grooves of the brain, and somehow improves abnormalities, and functions (Guo et al., 2008; Ding et al., 2005). Studies have shown that exercise improves brain resistance to injury and improves cognitive and motor processes (Radak et al., 2006; Spiegelman, 2013). Both structural and functional changes in the brain play an important role in improving memory and learning in response to exercise (Gordon, Benson, Bird, & Fraser, 2009). Since exercise induces neurogenesis in humans, it decreases the risk of Alzheimer’s (Henriksen, 2002), Parkinson’s and some other neurogenic diseases (Henriksen, 2002; Yuede et al., 2009). Exercise reduces blood glucose, and this decrease after exercise is associated with increased response of the cells to insulin and improved insulin function (Gordon, Benson, Bird, & Fraser, 2009). Previous studies have shown that exercise can increase the expression of GLUT proteins in skeletal muscle (Henriksen, 2002). Therefore, exercise via reducing blood glucose improves memory impairment by STZ. It has also been shown that exercise helps to change the antioxidant status of rats (Yuede et al., 2009).

Regular and continuous exercise has beneficial effects on the brain. In this way, exercise increases capillary growth, dendritic connections, and increases the processing efficiency of the central nervous system (Cotman & Berchtold, 2006). In addition, new studies have shown that regular exercise reduces brain damage after stroke (Carro, Trejo, Busiguina, & Torres-Aleman, 2001) and reduce the severity of many age-related illnesses. It has been shown, for example, that aerobic exercise prevents decrease in the amount of gray matter and mental ability in the age-old cortex (Colcombe, 2004; Blomquist & Danner, 1987; Evans et al., 1993). It has been mentioned that long-term exercise results in an increase in the level of antioxidants and antioxidant enzymes that can protect brain from oxidative damage for example, it has been shown that after long-term exercise the levels of Malondialdehyde
(MDA), a product of lipid peroxidation and oxidative stress biomarker, and ascorbic acid in the brain decreased and increased, respectively (Liu et al., 2000). Ascorbic acid as a nerve modifier, improves memory and learning disorders caused by Alzheimer’s (Harrison, Hosseini, Dawes, Weaver, & May, 2009).

BDNF (Brain Derived Neurotrophic Factor) is a growth factor that has protection effect on neurons. Also, many evidences showed the involvement of low BDNF in neurological disease such as Alzheimer’s disease and depression (Roghani, Balouchinejad, Khalili, & Mahdi, 2006).

Exercise through reducing the blood glucose helps to treat diabetes, and it is possible that with changes in the level of BDNF protein in the central and peripheral nerves in aerobic exercise regimens, it can reduce complications of diabetes. BDNF and its receptor tyrosin kinase B are essential for learning and memory in the hippocampus. Among the neurotrophic factors, BDNF has the most significant role in learning and memory processes and synaptic flexibility. Regular exercise for a long period of time has the best effect in reducing the spatial memory loss caused by diabetes. Thus, it can be considered that the positive effect of exercise in modulating the complications of diabetes depends on its continuity and regularity. (Mattson, Culmsee, Yu, & Camandola, 2000; Monti, Polazzi, & Contastabile, 2009).

5. Conclusions

According to the data of the recent study, it can be concluded that the use of ripe pistachio hulls hydro-alcoholic extract and aerobic training alone and simultaneously can improve passive avoidance memory in male diabetic rats. It might be considered as an adjuvant therapy with other traditional anti-diabetic medications. Nevertheless, further studies are necessary for elucidation the involvement of probable mechanisms of their effects.
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Conflict of interest statement

The authors declare that there is no conflict of interest in this work.
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