

⇒ Research Article



Antioxidant Effect of Swimming Training and Royal Jelly Consumption in the Hippocampus Tissue of Rats With Alzheimer's Disease

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Background: As an age-related disease, Alzheimer's disease (AD) is characterized by memory loss and cognitive impairment. Although oxidative stress factors increase in AD, exercise and nutrition can have protective and antioxidant effects. This study aimed to explore the antioxidant effects of swimming training (ST) and royal jelly (RJ) consumption in the hippocampus tissue of rats with AD.

Methods: In this experimental study, 20 rats with AD were selected and divided into four groups, including control, ST, RJ, and ST+RJ. In order to probe the effects of AD induction on superoxide dismutase (SOD) and glutathione peroxidase (GPx), five healthy rats were assigned into the healthy control group. For eight weeks, the ST groups performed ST three times per week (5 minutes in the first week to 60 minutes in the last week), and the RJ groups received 100 mg/kg RJ per day. The Shapiro-Wilk, independent samples *t* test, and two-way analysis of variance (ANOVA) tests were used to analyze the findings ($P \leq 0.05$).

Results: AD induction had a significant effect on increasing SOD ($P=0.04$) and decreasing GPx ($P=0.001$). Also, ST ($P=0.001$) and RJ ($P=0.01$) had a significant effect on increasing GPx; ST ($P=0.001$) and RJ ($P=0.001$) had a significant effect on decreasing SOD. Furthermore, ST with RJ had an interactive effect on increasing GPx ($P=0.03$) and decreasing SOD ($P=0.001$).

Conclusion: In general, ST and RJ appear to simultaneously improve the gene expression of antioxidants in the hippocampus tissue of AD rats. In addition, the antioxidant effects of ST were more favorable than those of RJ. It appears that different doses of RJ should also be considered.

Keywords: Royal Jelly, Swimming Training, Alzheimer's Disease, Antioxidant

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Background

Alzheimer's disease (AD), usually recognized by the gradual deterioration of intellectual and social functioning, memory loss and cognitive impairment, is characterized by the neurotoxic effect of amyloid beta ($A\beta$) oligomers (1). Patients with AD lose their memory and cognitive abilities through drastic changes in behavior.

The world's population is getting older day by day. It is estimated that there are 35 million dementia cases worldwide and is predicted to rise to 65 million cases by 2030 (2). A variety of factors are involved in the development of AD, including oxidative stress, lactate dehydrogenase, antioxidant levels, increased glutamate-dependent toxicity, decreased acetylcholine and serotonin levels, dopamine neurons decrease or loss, free radical-induced damage, and inflammation of the brain tissue due to the presence of inflammatory factors that contribute to the disease (3). In other words, researchers believe that in the pathology of AD, factors such as microglia activity, increased inflammatory cytokines, cerebrovascular disorders, mitochondrial neuronal

disorders with increased reactive oxygen species (ROS) and interactively increased $A\beta$ and neurofibrillary tangles cause hyperphosphorylation of the tau protein and cause synaptic damage (4). For example, the brain cells of people with dementia, including AD, show evidence of free radical damage. Free radicals appear to be one of the causes of amyloid accumulation in the brain, that is a characteristic of AD.

With the advancement of medical science, various methods have been reported for the prevention and treatment of AD. Although effective methods have not been proposed so far, recent studies have shown that exercise, as a non-invasive intervention, is effective in preventing the complications of AD. Also, improved memory and learning skills in animal models of the disease have been reported following long-term exercise (5). Exercise training (resistance, swimming, and running) seems to increase the antioxidant capacity, peripheral neurotrophins, blood flow to the nervous system, nitric oxide, the proteins responsible for nuclear transcription, memory and learning skills in the animal model of AD, and decrease beta amyloid levels and oxidative stress

(5, 6). Also, 12 weeks of moderate-intensity running training increased blood and brain glutathione levels and brain-derived neurotrophic factor, while it decreased inflammatory factors, oxidative damage, and C-reactive protein in young men (7). Exercise has been reported to reduce disease and symptoms, enhance hippocampal neurogenesis, and terminate memory loss in animal models (8). In this regard, it has been reported that six weeks of physical activity on the treadmill improves redox balance and also increases nerve growth factor (NGF) gene expression in the hippocampal tissue (9). In addition to exercise, it has been proven that administration of vitamins E and C can prevent the brain damage.

In addition to the beneficial role of exercise in diseases of the nervous system, researchers point to the role of proper nutrition and natural antioxidants. Royal jelly (RJ) is a bee product that has been traditionally used in European and Asian medical systems for a long time. It has a variety of pharmacological activities that may, for example, prevent aging, and is anti-inflammatory, antioxidant, antihypertensive, and anti-hyperglycemic (1). RJ seems to improve insulin resistance in AD patients because it has insulin-like activity. In addition, researchers suggested that RJ leads to differentiation of brain stem cells and increases neurotrophin transcription. Moreover, it neutralizes free radicals such as anion oxide in the brain of animal models of AD (10). In this regard, the researchers showed that 100 and 200 mg per kilogram of body weight consumption of RJ for 14 days can increase memory, learning, and neurotrophins and reduce oxidative damage in the brain tissue of intraventricularly-injected streptozotocin-induced AD rats (10). Also, in a review study by Ali and Kunugi in 2020, the use of RJ improved the function of the nervous system, memory, and learning, while it decreased inflammatory factors, oxidative stress, and beta amyloid (11).

Although some studies have examined the desired effects of each of these interventions, no study has been conducted to examine the interactive effect of both factors on antioxidant gene expression levels (12). However, the interaction of training on different slopes and the use of RJ improved motor balance (12) and decreased gene expression of A β and γ -secretase (13) in rats with AD. On the other hand, swimming training (ST) with RJ had no interactive effect on increasing BDNF and NGF in rats with AD (14). Hence, the interactive effects of these two interventions are not yet well understood.

Objective

RJ has anti-aging and neural activity that decreases amyloid plaque pathology in APP/PS1 rats (15). Given the potential effects of ST and RJ on preventing AD progress, this study attempted to explore the effect of ST and RJ on superoxide dismutase (SOD) and glutathione peroxidase (GPx) in trimethyltin (TMT)- induced AD rats.

Material and Methods

In this experimental study, 25 healthy male Sprague Dawley rats with an age range of 8-10 weeks and weight range of 200-220 g were purchased and transferred to animal laboratory for 1 week to adapt under standard conditions. They were maintained in autoclavable transparent polycarbonate cages, optimum temperature (20 to 24°C), relative humidity of about 55 to 65%, 12-hour darkness-light cycle, and free access to water and food. Then, on the eighth day, 20 rats were injected intraperitoneally with 8 mg/kg TMT (16). Upon assuring the complete effect on the hippocampus after four days, the rats with AD were randomly divided into four equal groups, including control, ST, RJ, and ST+RJ. To probe the effects of AD induction on SOD and GPx levels, five rats were assigned into the healthy control group.

All ethical principles of working with animals in this study were supervised by the Committee on Ethics of Working with Laboratory Animals, Islamic Azad University, Marvdasht Branch, Iran. The ST groups performed training in rodent bathtub for 8 weeks, 3 sessions per week. The rats were trained for 5 minutes in the first week of ST, and by the end of the study period, the time was 60 minutes in the eighth week (17). Also, the rats in RJ groups received 100 mg/kg RJ daily for eight weeks. In this study, to use RJ daily, 250 mg of RJ was immediately removed from the temperature of -21°C with a scalpel and dissolved in 3 mL of normal saline. Immediately after ensuring their dissolution by shaker, the RJ groups were injected with 0.3 mL of RJ solution to each rat (12, 13, 18). Next, 48 hours after the last training session, the rats were anesthetized with 10% ketamine (50 mg/kg body weight) and 2% xylazine (10 mg/kg body weight) after about 5 minutes. The hippocampal tissues were then extracted by specialists and placed in cryotube in liquid nitrogen and stored at -70°C for further investigation. SOD and GPx were measured by real time polymerase chain reaction (PCR) method.

Quantitative Reverse Transcription Polymerase Chain Reaction Method

To measure the expression levels of myogenin and myonectin after homogenization of the hippocampal tissue, RNA was extracted according to the company protocol (Kiagen, Germany); and spectrophotometric method of light absorption at 260 nm was used to evaluate the purity of RNA. Then, after extracting RNA with the desired purity and concentration, cDNA was synthesized according to the protocol of the manufacturer (Fermentas, USA) and the synthesized cDNA was used to perform the reverse transcription reaction.

First, all the designed primers (Table 1) related to all genes were examined, and then the expression of genes was studied using a relative quantitative method based on the difference between Ct and Quantitative reverse transcription PCR (RT q-PCR).

The quantification of the levels of the variables

Table 1. Sequence of Forward and Reverse Primers of SOD, GPx, and B2m Genes for Real Time PCR Reaction

Gene	Forward (5'-3')	Reverse (5'-3')	Product Size (bp)
B2m	5'-CGTGCTTGCCATTCAGAAA-3'	5'-ATATACATCGGTCTCGGTGG-3'	244
SOD	5'-CAAGGAACCACAGGCCTTAT-3'	5'-GGCTAACATTCTCCAGTTGA-3'	133
GPx	5'-CATTGAGAATGTCGCGTCCC-3'	5'-TTGCCATTCTCCTGATGTCGG-3'	141

was performed using the formula $2^{-\Delta\Delta CT}$. The relative expression of the genes was compared using an internal control gene called beta-2 microglobulin (B2m) and then its expression was compared to the gene in the healthy control group. The primers used were also designed using the PubMed site, and the efficiency of the primer was evaluated using the software available on the site.

Data Analysis Procedure

The Shapiro-Wilk test was used to examine the normal distribution of data and independent sample t-test and two-way analysis of variance (ANOVA) test were used to analyze the data in SPSS software version 22 ($P \leq 0.05$).

Results

The levels of SOD and GPx in the hippocampal tissue of rats are reported in Figures 1 and 2, respectively. The results of independent samples *t* test showed that induction of AD by TMT had a significant effect on increasing SOD ($P=0.04$), as well as decreasing GPx ($P=0.001$) in the hippocampal tissue of rats (Table 2).

The results of two-way ANOVA showed that eight weeks of ST ($F=16.86$, $P=0.001$ and effect size=0.51) and RJ ($F=8.03$, $P=0.01$ and effect size=0.33) had a significant effect on increasing GPx in the hippocampal tissue of rats with AD. Also, ST with RJ had interactive effects on increasing GPx in the hippocampal tissue of rats with AD ($F=5.60$, $P=0.03$ and effect size=0.25). In addition, eight weeks of ST ($F=20.14$, $P=0.001$ and effect size=0.55) and RJ ($F=29.00$, $P=0.001$ and effect size=0.64) had a significant effect on the reduction of SOD in the hippocampal tissue of rats with AD. Besides, ST with RJ had interactive effects on reducing SOD in the hippocampal tissue of rats with AD ($F=17.83$, $P=0.001$ and effect size=0.52).

Discussion

This study aimed to investigate the antioxidant effects of ST and RJ in the hippocampal tissue of rats with AD. The results showed that induction of AD by TMT significantly increased SOD and decreased GPx gene expression in the hippocampal tissue of rats. Due to the need for simulations of AD, the use of the neurotoxin TMT as one of the common methods of inducing AD has been considered by some researchers. Trimethyltin chloride is a neurotoxic substance that selectively causes neuronal death in the limbic system, especially the hippocampus. Evidence shows that TMT alters the concentration of neurotransmitters involved in memory, such as acetylcholine and glutamate and causes memory

impairment by causing oxidative damage (19). On the other hand, it seems that TMT-affected neurons may react in two ways, one is the mechanism of oxidative stress, mediated by non-NMDA receptors, and the other is the activation of the NMDA glutamate receptor. The NMDA

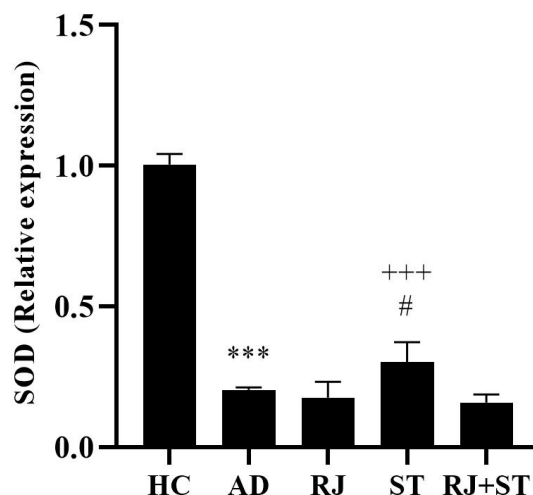


Figure 1. SOD Gene Expression Levels in the Hippocampal Tissue of Rats in the Research Groups. HC: Healthy Control, AD: Alzheimer's Disease, RJ: Royal Jelly, ST: Swimming Training, RJ + ST: Royal Jelly + Swimming Training. *** ($P = 0.001$) Significant decrease in the AD group compared to the HC group. # ($P = 0.05$) Significant increase in the ST group compared to the AD group. +++ ($P = 0.001$) Significant increase compared to the RJ and ST groups.

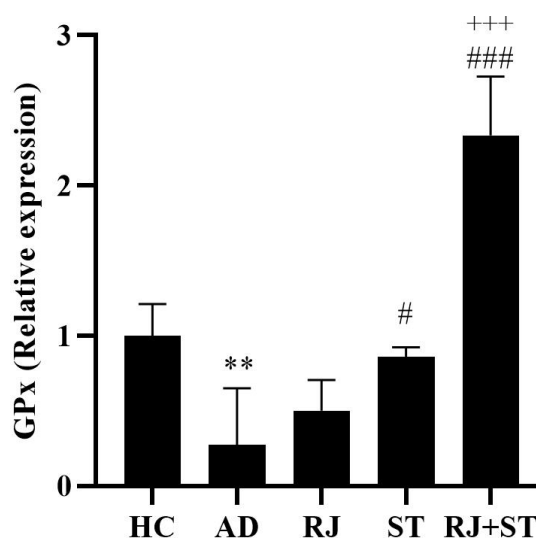


Figure 2. GPx Gene Expression Levels in the Hippocampal Tissue of Rats in the Research Groups. HC: Healthy Control, AD: Alzheimer's Disease, RJ: Royal Jelly, ST: Swimming Training, RJ + ST: Royal Jelly + Swimming Training. *** ($P = 0.001$) Significant decrease in the AD group compared to the HC group. # ($P = 0.05$), ### ($P = 0.001$) Significant increase compared to the AD group. +++ ($P = 0.001$) Significant increase compared to the RJ and ST groups.

Table 2. Results of Two-Way ANOVA and Independent Samples T Test for Investigating the Effect of ST and RJ on Research Variables

Parameter	Independent Sample <i>t</i> test		Two-way ANOVA Test								
	T	P	ST			RJ			Interactive effects		
			F	P	Effect Size	F	P	Effect Size	F	P	Effect Size
GPx	- 4.92	0.001	16.86	0.001	0.51	8.03	0.01	0.33	5.60	0.03	0.25
SOD	1.55	0.04	20.14	0.001	0.55	29.00	0.001	0.64	17.83	0.001	0.52

TMT: Trimethyltin; GPx: Glutathione peroxidase; SOD: superoxide dismutase; RJ: royal jelly; ST, swimming training. Significant level set at $\alpha=0.05$

glutamate receptor response increases the oxidative stress effect and accelerates neuronal death, but in the presence of TMT, nitric oxide levels may also increase and react with superoxide anions to produce toxic peroxide nitrate anions (20). In general, the cell response to increased oxidative stress following this neurotoxin has not been well studied, so that following exposure to the neurotoxin TMT in Purkinje cells, the path of cell death and increased oxidative stress was not observed (21). Therefore, it seems that the increase in SOD following TMT poisoning can be attributed to the compensatory response of the antioxidant system to counteract it. Furthermore, there are many unknown pathways following poisoning with this neurotoxin.

In this study, RJ consumption had a significant effect on increasing GPx and decreasing SOD gene expression in the hippocampal tissue of rats with AD. Consistent with the findings of the present study, seven days of consumption of 85 mg/kg RJ resulted in a significant increase in GPx, as well as a decrease in SOD in rats exposed to cadmium chloride intoxication (22). Also, RJ consumption significantly improved GPx, SOD, and catalase in rats with varicocele (23). RJ has been reported to be rich in vitamins B, C, D, and E, as well as minerals, especially potassium. RJ has many medicinal properties, such as antioxidant, anti-inflammatory, anti-tumor, and antibiotic activities (24). Therefore, RJ might alleviate the oxidative damage induced by AD induction with TMT toxin in the hippocampal tissue due to its antioxidant and anti-apoptotic activities by enhancing the activity of the body's antioxidant defense system and reducing lipid peroxidation, as well as inhibiting apoptosis.

Also, in the present study, eight weeks of ST significantly increased GPx and reduced SOD gene expression in the hippocampal tissue of rats with AD. Concerning the antioxidant effects, eight weeks of high intensity interval training resulted in a significant increase in GPx and catalase (25). Eight weeks of resistance training led to a significant improvement in the protein levels of catalase and SOD in diazinon-poisoned rats (26). Also, two weeks of ST improved GPx protein levels in rats. One of the reasons that the findings of the present study are consistent with the previous study may be the same duration of exercise activity. Studies show that exercise with the mechanism of increasing mitochondrial biogenesis, increasing nuclear transcription proteins such as NRF1/2, and activating the AMPK pathway and protein kinases

increases the expression of antioxidants, cell metabolism, eNS, BDNF, improves cognitive function, and decreases ROS and neuronal inflammation (3, 27). Alternatively, RJ with the mechanism of N-terminal kinase c-Jun, having non-enzymatic antioxidants such as vitamins A, E, and D, inhibits ROS and neuroinflammation, and by activating the cAMP/p-PKA/p-CREB/BDNF pathway, inactivates APP and γ -secretase, and reduces oxidative stress (23).

According to the studies, the intensity of physical activity seems to be an important factor affecting antioxidant enzymes changes, so that in most studies whose findings indicate the antioxidant effects of exercise, the physical activity applied was of high intensity. However, it should be noted that antioxidant responses to acute and vigorous physical activity are different from long-term physical activity, so that acute and vigorous physical activity may increase oxidative stress; but regular and long-term exercise activities can decrease oxidative stress by increasing antioxidant defense (27). Concerning the response of antioxidants to exercise, it is likely that along with increased production of free radicals, adaptations in the rate of production and activity of the enzymatic antioxidant system of cells occur, which counteract adverse effects. Increased oxidative enzyme activity is also known to be an adaptation to endurance training, that makes GPx more widely used in the body's antioxidant defense system, but the mechanisms of these two approaches have not yet been elucidated; however, these seemingly inconsistent adaptations are among the changes occurring with sports activities (27). Studies show that exercise with the mechanism of increasing mitochondrial biogenesis, increasing nuclear transcription proteins such as NRF1/2, and activating the AMPK pathway and protein kinases, can increase the expression of antioxidants, cell metabolism, eNS, BDNF, improve cognitive function, and decrease ROS and neuronal inflammation (3,28). Alternatively, RJ with the mechanism of N-terminal kinase c-Jun, having non-enzymatic antioxidants such as vitamins A, E, and D, inhibits ROS and neuroinflammation, and by activating the cAMP/p-PKA/p-CREB/BDNF pathway inactivates APP and γ -secretase, and reduces oxidative stress (29, 30).

There is limited information about the interactive effect of exercise and RJ and their antioxidant effect; however, exercise and RJ interactively increased serotonin gene expression levels (3), motor balance (12), and memory and learning (1) in rats with AD.

Concerning the interactive effects, the present study showed that ST along with RJ had interactive effects on increasing GPx and decreasing SOD gene expression levels. These findings indicate that exercise and nutrition can be two effective antioxidant factors in ameliorating the disorders due to free radicals in AD. Regarding the effects of exercise activities, the studies showed that exercise activities alter the antioxidant enzymatic activity of the tissues according to the type of exercise protocol, the volume of exercise, and the existence of rest periods between training programs. On the other hand, the presence of RJ with antioxidant and anti-apoptotic activities can strengthen the antioxidant defense system. Therefore, it seems that in the present study, RJ consumption and ST with distinct mechanisms have led to an improvement in the antioxidant status in the hippocampal tissue of rats with AD.

Considering the fundamental differences in the activity levels of antioxidant enzymes compared to the levels of gene expression, it seems that the lack of measurement of these antioxidants by ELISA methods and even the study of hematoxylin-eosin method is one of the limitations of this study. Therefore, it is suggested that in future studies, the levels of these variables be measured by different methods. In addition, due to the effect of neurotrophins and the cAMP/p-PKA/p-CREB/BDNF signaling pathway in both interventions, the lack of measuring these variables and this pathway in this study is another limitation. Therefore, it is suggested that in future studies, more molecular cell studies be performed following TMT poisoning and nutritional and exercise interventions.

Conclusion

ST and RJ appear to have simultaneously beneficial effects on nervous system disorders, and these favorable effects are more evident in regular swimming training. Thus, the use of these two interventions is recommended for patients with cognitive disorders.

Authors' Contribution

Laboratory studies and tests: A H; study and review: KH M and SA H; analysis and interpretation of data: A H, KH M and SA H.

Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical Approval

Researchers received introduction letters from Islamic Azad University of Marvdasht, Iran.

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