ORIGINAL ARTICLE

Effects of endurance training on hippocampus DJ-1, cannabinoid receptor type 2 and blood glucose concentration in diabetic rats

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ABSTRACT

Aims/Introduction: To investigate the effect of endurance training on hippocampus DJ-1 and cannabinoid receptor type 2 (CB_2) protein and blood glucose concentration in diabetic rats.

Materials and Methods: A total of 32 rats were randomly divided into diabetic (D), diabetic and exercise (DE), exercise (E) and control (C) groups. The endurance training was carried out five times per week for 6 weeks. The hippocampus DJ-1 and CB_2 were measured using an enzyme-linked immunosorbent assay method.

Results: The level of DJ-1 in the D group was significantly higher than the other groups ($P \leq 0.01$). However, the level of DJ-1 was not significantly different between the C, E and DE groups. In addition, the level of CB_2 was significantly lower in the D group compared with the other groups ($P \leq 0.01$). Blood glucose was significantly higher in the D group compared with the DE group ($P \leq 0.05$). Furthermore, a significant positive correlation between the level of DJ-1 and blood glucose was observed ($r = 0.67$, $P \leq 0.001$). There was also a significant inverse correlation between the level of CB_2 and blood glucose ($r = -0.77$, $P \leq 0.001$).

Conclusions: The results of this study suggest that the level of DJ-1 and CB_2 might change in response to diabetes, and regular aerobic exercise could mediate the effect of DJ-1 and CB_2 on diabetes-induced neurodegenerative diseases.

INTRODUCTION

The gradual and progressive loss of neural cells in neurodegenerative diseases leads to nervous system dysfunctions[1–3]. According to the National Institute of Neurological Disorders and Stroke, approximately 50 million Americans are affected each year from more than 600 neurological disorders[1]. Several factors are associated with neurodegenerative diseases, including α-synuclein, parkin, PINK1, dardarin (leucine-rich repeat kinase 2) and DJ-1[4]. Protein deglycase DJ-1 is a widely distributed and highly expressed protein in the brain and extracerebral tissues[4]. Although the exact mechanism and roles of DJ-1 are not fully known, it is believed that DJ-1 is associated with metabolic syndrome[5], and various cellular processes including response to oxidative stress, transcriptional regulation, mitochondrial regulation, ribonucleic acid-binding, androgen-receptor signaling, spermatogenesis and fertilization[4,6]. In

addition, DJ-1 has been shown to play an important role in neuronal dynamics and modulation of neurodegeneration by reducing oxidative stress, inflammation and neurotoxicity[7]. A higher concentration of DJ-1 has been observed in response to a high level of glucose in renal glomerular mesangial cells and β-cells of the islets of Langerhans[1,6]. An abnormal increase in blood glucose has been reported in most neurodegenerative diseases, such as Alzheimer's disease[8], Parkinson's disease[9] and Huntington's disease[10], and dysfunction of DJ-1 has also been reported in diabetes mellitus in a previous study[6].

Cannabinoid receptors (CBs) are a cluster of receptors that exist in all tissues of the body and are involved in various physiological processes, including apoptosis, pain reduction and memory regulation[11,12]. These receptors include two isoforms: cannabinoid receptor type 1 (CB_1), and cannabinoid receptor type 2 (CB_2). CB_2 is expressed in most tissues of the central nervous system[13] and is believed to be involved in nerve-protecting mechanisms[14]. It has been shown that CB_2 modifies the

side-effects of neurodegenerative diseases, and improves brain function by modulating the production of amyloid plaques and reduction of the destructive effects of inflammatory reactions and oxidative stress[15]. The negative correlation between the long-term increase in blood glucose and CB_2 suggests that hyperglycemia might decrease the level of CB_2 in the nervous system[13]. Therefore, it seems plausible that the levels of DJ-1 and CB_2 might change in neurodegenerative diseases or diabetes.

Diabetes mellitus is defined as a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action or both[6,16], and had been shown to cause several health complications including cardiovascular diseases, retinopathy, nephropathy and neuropathy[17]. High blood glucose has harmful effects on specific areas of the brain, such as the hippocampus, and could cause memory loss, learning and problem-solving difficulties, and mental and motor disorders[8]. Increased blood glucose is one of the major mechanisms involved in nerve degeneration, which has been observed in most neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease[18].

Medications used to control diabetes and subsequent conditions might have moderate-to-severe side-effects[19,20]. Therefore, non-pharmacological treatments are preferred by neuroscientists and other health professionals. A change in lifestyle and increase in daily energy expenditure is the most common non-invasive treatment for diabetes patients[20–22]. Previous studies have shown that physical activity increases the metabolism at rest and during activity, resulting in positive changes in blood glucose[23,24]. In addition, physical activity is likely to prevent diabetes-related nerve damage[25,26] by modulating inflammatory responses[27], improving anti-oxidant responses[28] and decreasing blood glucose levels[29].

The effect of exercise on diabetes has been widely studied[24,30]. However, the mechanisms for the effect of exercise on the modulation of nerve damage from diabetes is not clear[31]. Given the important role of DJ-1 and CB_2 in neurodegeneration, the aim of the current study was to investigate the effect of endurance training on the level DJ-1 and CB_2 in diabetic rats.

METHODS
A total of 46 male Wistar rats (aged 8–10 weeks) were kept in groups of three in polycarbonate cages in a room with an average temperature of $22 \pm 2°C$ and dark–light cycles of 12:12 h. Animals were handled by one researcher at the animal house of Lorestan University of Medical Sciences, and had free access to water and standard rat food (Pars Khorakdam Co, Tehran, Iran). All experiments in the current study followed the ethical principles approved by the Animal Ethics Committee of Lorestan University (reference number: LU. ECRA. 2017.1).

Rats were randomly, but unequally, assigned into diabetic (D; $n = 13$), diabetic and exercise (DE; $n = 13$), exercise (E; $n = 10$) and control (C; $n = 10$) groups. The number of rats was higher in the D and DE groups to account for diabetes-induced mortality. Exercise familiarization in the E and DE groups included walking on a treadmill for 10–15 min at 5–10 m/s five times before the start of the experiment.

Diabetes was induced after 12 h of food deprivation by intraperitoneal injection of streptozotocin (STZ) solution (Sigma, St. Louis, MO, USA), 50 mg/kg dissolved in 0.5 mol/L fresh citrate buffer, pH 5.0[32]. Rats in the E and C groups received an equivalent volume of citrate buffer. Two rats in the DE group died within 48 h after the injection of STZ solution. 48 h after inducing diabetes, blood samples were collected from the tail of the remaining rats and analyzed using the glucometer (Roche Diagnostics K.K., Tokyo, Japan). Rats with a blood glucose level >300 mg/dL were considered diabetic and were included in the study. Given that blood glucose levels in all injected rats were >300 mg/dL, diabetes was confirmed in all rats in the D and DE groups. Blood glucose levels were measured every 14 days to monitor changes in blood glucose during the intervention period (Figure 1).

Rats in the E and DE groups were trained on a treadmill (Azarakhsh animal treadmill; Pishro Andisheh Technology Engineering Co., Tehran, Iran) at the intensity of 50–55% of maximum aerobic capacity[33]. Both groups completed five training sessions per week for 6 weeks, and every training session started with 3 min of warm-up and was completed with 3 min of cool down. Intensity and volume training sessions increased gradually over the intervention period. In the first week, training consisted of 10 min of running on a treadmill at 10 m/min. In the second week, training duration increased to 20 min, while the intensity remained at 10 m/min. In the third week, training increased to 20 min of running at 15 m/min. In week 4, 5 and 6, rats trained for 30 min at 15, 18 and 18 m/min respectively. When rats failed to maintain the training intensity, they were encouraged to run by tapping on a Perspex box, and as necessary, electrical stimulation through a metal grid at the end of the treadmill. Rats received a maximum of 10 shocks of 0.5-s duration over the exercise period. Rats in the E and DE groups did not show any signs of severe fatigue or locomotor impairment during the intervention period. However, a previous study has suggested that severe fatigue increases blood lactate levels and might increase the side-effects of diabetes[34].

Blood glucose was measured before STZ injection, 48 h after STZ injection, and at the end of week 1, week 3 and week 5, and 24 h after completion of the study in week 6. For the accuracy of blood glucose analysis, access to food was restricted 12 h before blood glucose assessment.

Body mass of rats was measured at the beginning of the study and the end of week 2, week 4 and week 6, using a digital scale with the accuracy of 0.001 kg (Seca, Hamburg, Germany). Body mass measurements were completed in the following week of every blood glucose assessment to reduce the effect of food deprivation and stress of blood sampling on body mass.
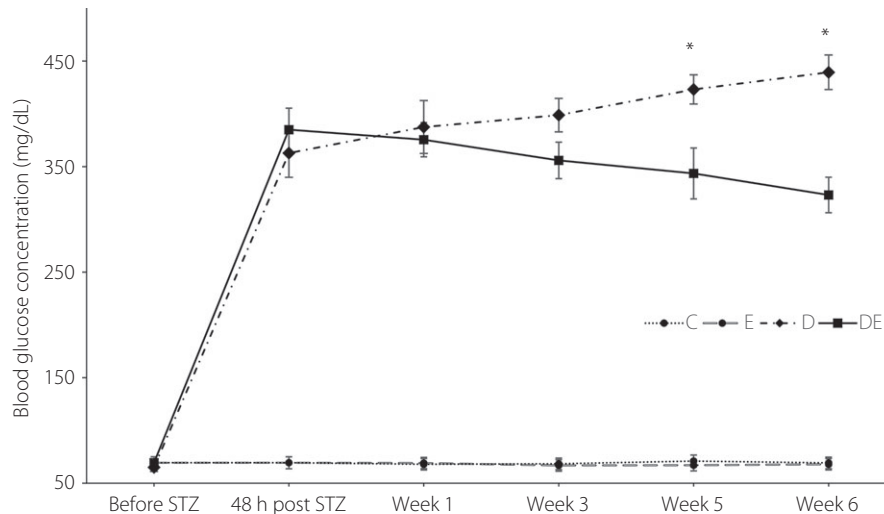
Figure 1 | Blood glucose levels during the intervention. *Significantly different from the diabetic and exercise (DE) group. C, control group; D, diabetic group; E, exercise group; STZ, streptozotocin.

On completion of the intervention period, rats were anesthetized by intraperitoneal injection of ketamine and xylazine, 24 h after the last exercise session. The hippocampus was stored in a −80°C freezer for further analysis. For the analysis of DJ-1 and CB_2, 100 mg of tissue was rinsed with 1× phosphate-buffered saline, homogenized in 1 mL of 1× phosphate-buffered saline and stored overnight at −20°C. Two freeze–thaw cycles were carried out to break the cell membranes, and the homogenates were centrifuged for 5 min at 5,000 g at 2–8°C. The supernatant was removed and assayed immediately. DJ-1 and CB_2 protein levels in the hippocampus were measured using an enzyme-linked immunosorbent assay kit (Cusabio DJ-1 kit with a sensitivity of 0.078 ng/mL; Tokyo, Japan; and Biomatik CB_2 kit with a sensitivity of 0.058 ng/mL; Cambridge, Canada). Antibodies specific for DJ-1 and CB_2 molecules were pre-coated on a microplate. Standards and samples were pipetted into the wells, and any target molecule present was bound by the immobilized antibody. After removing any unbound substances, biotin-conjugated antibodies specific for DJ-1 and CB_2 molecules were added to the wells. After washing, avidin-conjugated horseradish peroxidase was added to the wells. After a wash to remove any unbound avidin enzyme reagent, a substrate solution was added to the wells and color developed in proportion to the intensity of the target molecules bound in the initial step. The color development was stopped and the intensity of the color was measured. All measurements were carried out in duplicates and the average value was used for statistical analysis.

Five rats from the D group and two rats from the DE group died during the experiment. Three rats (one from the DE group and two from the E group) did not complete 85% of the duration of the overall training sessions time and were excluded from the study. On completion of the study, eight rats from the DE group, and eight rats from the D and E groups satisfied the training and diabetes requirements, and were included in the study. To achieve an equal number of rats in each group, two rats from the C group were randomly excluded at the end of the study.

Data were analyzed using SPSS 25.0 (SPSS Inc., Chicago, IL, USA) and reported as mean ± standard deviation. The normality and homogeneity of data were analyzed using the Shapiro–Wilk and Levene's test, respectively. A one-way analysis of covariance (ANCOVA) was used to compare the level of hippocampus DJ-1 and CB_2 of rats in the D, DE, E and C groups. The body mass of rats at the end of the study was selected as a covariate to partial out the effect of rats' body mass on the level of DJ-1 and CB_2. The correlation between DJ-1, CB_2 and blood glucose was assessed with a bivariate Pearson correlation coefficient.

A within-between (groups × time) repeated measures ANOVA was used to compare differences in bodyweight and blood glucose between groups through the study period. The probability level of statistical significance was set at $P ≤ 0.05$. Effect sizes were calculated using partial eta squared (η_p^2) with values of 0.2, 0.6 and >1.2 considered to be a small, medium and large effects[35]. A Bonferroni post-hoc test was used to compare the difference between groups.

RESULTS

The concentration of hippocampus DJ-1 is presented in Figure 2. The ANCOVA showed that, after accounting for the effects of bodyweight, there was a statistically significant effect of condition on the level of DJ-1 at the end of the 6-weeks intervention ($F_{3,27} = 7.84$, $P = 0.001$, $\eta_p^2 = 0.466$). The results of the Bonferroni post-hoc test are shown in Figure 2.

In addition, after accounting for the effects of bodyweight, there was a statistically significant effect of condition on the
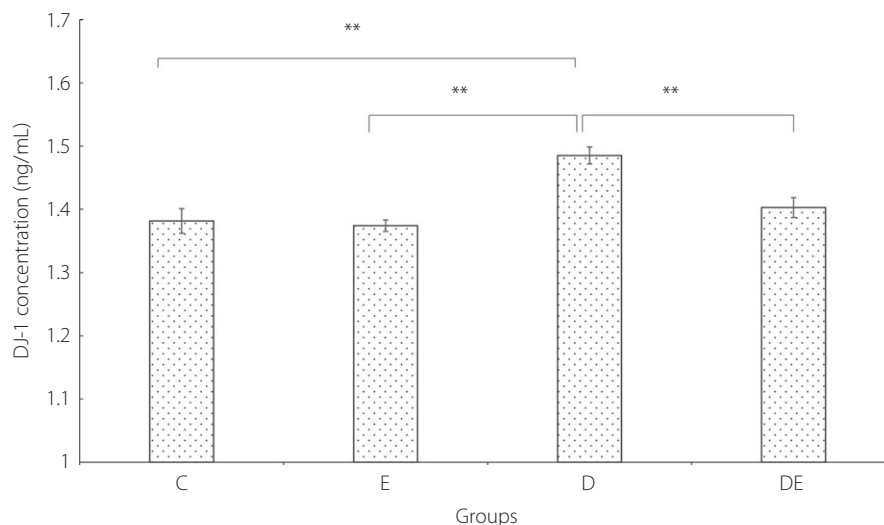
Figure 2 | The concentration of DJ-1 in the hippocampus of rats. *Significantly different, $P \leq 0.05$. **Significantly different, $P \leq 0.01$. C, control group; D, diabetic group; DE, diabetic and exercise group; E, exercise group.

Figure 3 | The concentration of cannabinoid receptor type 2 (CB_2) in the hippocampus of rats. *Significantly different, $P \leq 0.05$. **Significantly different, $P \leq 0.01$. C, control group; D, diabetic group; DE, diabetic and exercise group; E, exercise group.
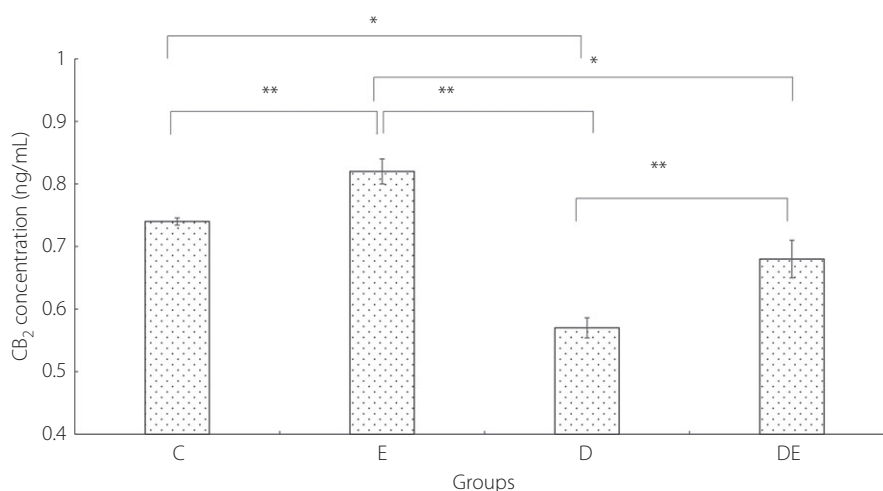
level of CB_2 at the end of the 6-week intervention ($F_{3,27} = 10.10$, $P < 0.001$, $\eta_p^2 = 0.550$). The results of the Bonferroni post-hoc test are shown in Figure 3.

A 4×6 mixed model ANOVA was used to investigate the impact of diabetes, exercise, and the combination of diabetes and exercise on blood glucose during a 6-week study. In the current study, the blood glucose was measured six times (Figure 1). There was a significant main effect for time ($F_{3.25,91.11} = 7,130.97$, $P < 0.001$, $\eta_p^2 = 0.824$). There was also a significant effect of group ($F_{3,28} = 328.80$, $P < 0.001$, $\eta_p^2 = 0.972$). The D group showed significantly higher blood glucose than the DE ($P = 0.21$), E ($P < 0.01$) and C ($P < 0.01$)

groups. Both the E and C groups showed significantly lower blood glucose than the DE group ($P < 0.001$). The difference in blood glucose between the C and E groups was not statistically significant ($P = 1.00$). There was also a significant interaction of group and time ($F_{3,28} = 75.61$, $P < 0.001$, $\eta_p^2 = 0.890$).

A 4×4 mixed model ANOVA was used to investigate the impact of diabetes, exercise, and the combination of diabetes and exercise on bodyweight of rats during the 6-week study. Changes of body mass are presented in Figure 4. There was no significant main effect for time ($F_{2.45,68.52} = 2.216$, $P = 0.106$, $\eta_p^2 = 0.073$). There was also a significant effect of group ($F_{3,28} = 10.44$, $P < 0.001$, $\eta_p^2 = 0.528$). Both the D and DE
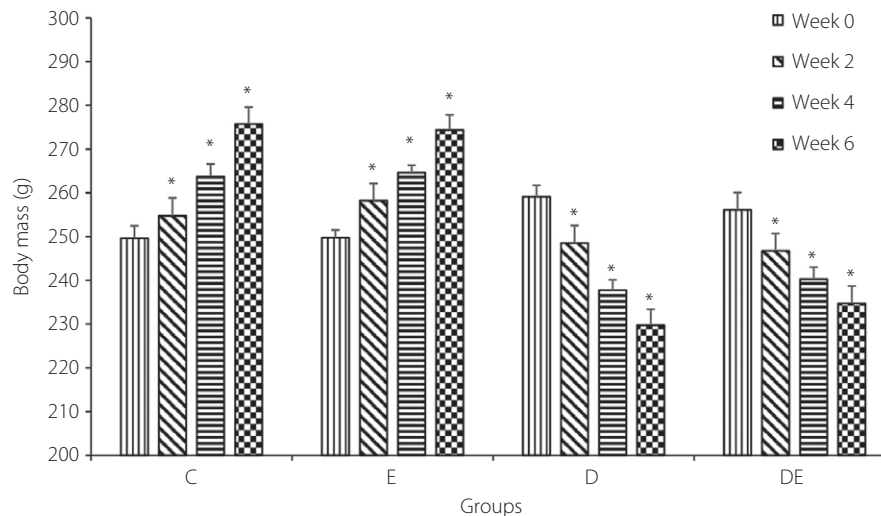
Figure 4 | Average body mass of rats in the four groups. *Significantly different from week 0. C, control group; D, diabetic group; DE, diabetic and exercise group; E, exercise group.

groups showed a significantly higher body mass than the E and C groups ($P < 0.05$). There was no significant difference between the bodyweight of the D group and the DE group, and between the E group and the C group. There was also a significant interaction of group and time ($F_{7.34,68.52} = 77.46$, $P < 0.001$, $\eta_p^2 = 0.892$).

Analysis of the results also showed a significant correlation between the level of DJ-1 and blood glucose in week 6 ($r_{30} = 0.67$, $P < 0.001$). There was also a significant inverse correlation between the level of CB_2 and blood glucose in week 6 ($r_{30} = -0.77$, $P < 0.001$). In addition, there was a significant inverse correlation between the level of CB_2 and DJ-1 in week 6 ($r_{30} = -0.62$, $P < 0.001$).

DISCUSSION

Previous studies have reported an increase in the level of DJ-1 and a decrease in the level of CB_2 in response to an abiding increase in blood glucose[13,36]. The effect of regular physical activity on the level of DJ-1 and CB_2 in diabetes patients is not fully understood. However, studies have shown an increase in the level of DJ-1 in response to lifestyle intervention, and have suggested that the increase in DJ-1 could prevent metabolic syndrome[5].

DJ-1 functions as a cytoplasmic redox-dependent protein chaperone[37], and the findings of recent studies suggest that DJ-1 might be associated with cancers and neurodegenerative diseases[38–41]. The role and mechanisms of the effect of DJ-1 in neurological disorders are not fully understood, and the findings of previous studies on the effect of DJ-1 concentration on neurodegenerative diseases are inconsistent. Some studies have shown a significant increase in levels of DJ-1 in patients with Parkinson's disease and Alzheimer's disease[2], whereas other studies reported lower levels of DJ-1 and α-synuclein in Parkinson's disease patients than in healthy people and those with Alzheimer's disease[42].

In the current study, the level of DJ-1 in the D group was significantly higher than the other three groups, suggesting that the levels of DJ-1 in male rats might have increased 6 weeks after induced diabetes. This finding supports the results of previous studies that showed an increase in DJ-1 in response to oxidative stress and hyperglycemia[1,43]. The significant difference between the levels of DJ-1 in the D group and DE group suggests that regular physical activity might have mediated the level of DJ-1 in diabetic rats. The non-significant difference between the levels of DJ-1 in the E and C groups could suggest that exercise might have regulated the levels of DJ-1 by reducing the levels of blood glucose in diabetic rats, but had no significant effect on the DJ-1 levels in healthy rats. The significant positive correlation between the levels of DJ-1 and blood glucose in the current study support this claim that increased levels of blood glucose might increase the level of DJ-1. This increase in the level of DJ-1 could be controlled by regular endurance training, possible by reducing the level of blood glucose.

The results of the present study also showed a significantly lower level of CB_2 in the D group compared with the other groups. The mechanisms involved in reducing CB_2 in diabetic rats are not yet clear; however, insufficiency of insulin function and increased levels of TGF-β1 could have contributed to the reduced levels of CB_2 in diabetic rats[13]. Barutta et al.[13] showed that the level CB_2 protein in diabetes patients with advanced nephropathy decreased in human and animal models as a result of increased levels of TGF-β1. Additionally, Oddi et al.[12], with induction of hemicerebellectomy in rats and injection of JWH-015 (CB_2 agonist) into cerebellar astrocytes, concluded that injection prevented the increase of nitric oxide synthase expression in astrocytes, reduced the oxidative stress in the neurons, increased levels of anti-oxidative (Hsp70) and anti-

apoptotic (Bcl-2) proteins, and prevented delayed apoptosis by signaling through the phosphoinositide 3-kinase–protein kinase B pathway. Palazuelos *et al.*[44] investigated the expression of CB_2 in activated microglia cells in the brain of Huntington's mice and showed the upregulation of this receptor, and this phenomenon is compatible with inflammatory responses at the onset of the disease.

The results of some studies in other neurodegenerative disorders were not consistent with the results of the present study. For example, by examining the post-mortem brains from patients with Alzheimer's disease, Benito *et al.*[45] found that CB_2 receptors are abundantly and selectively expressed in neuritic plaque-associated astrocytes and microglia, respectively, in the hippocampus and endothelial cortex sections. These contradictions can be related to the different types of disease, protein measurement method, the antibody used to measure CB_2 and extracted tissue type. The results of the current study showed a significant difference between the levels of CB_2 in the D and DE groups. This significant difference suggests that the negative effect of high blood glucose on the concentration of CB_2 could be reduced by regular endurance training. The significant inverse correlation between blood glucose and the level of CB_2 in the current study supports this possibility.

Regular exercise might also have a positive effect on the health of the nervous system[26,46]. The results of a previous study showed an improvement in brain functions after regular endurance training, especially in the hippocampus region[46]. The authors suggested that exercise might have improved the resistance to neurodegeneration caused by diabetes. In addition, some researchers showed an improvement in memory, a reduction of free electron levels, and an increase in the nerve growth factor and brain-derived neurotrophic factor levels after regular exercise[26,47,48]. Aksu *et al.*[49] reported that endurance exercise increases superoxide dismutase anti-oxidant enzyme levels in different parts of the brain, such as the hippocampus, and leads to an increase in the anti-oxidant capacity of the brain.

Some studies have been carried out to identify the mechanisms through which the beneficial results of exercising in improving the neurodegeneration in the brain could be understood. Some of these mechanisms include reduction of oxidative stress and inflammatory factors, improvement of the anti-oxidant defense, increase in angiogenesis, increase in secretion of neurotrophin and catecholamine, and neurogenesis, especially in hippocampus region[50–52]. Therefore, it seems that among the mechanisms related to exercise, reducing oxidative stress, improving anti-oxidant defense, and reducing inflammatory factors involved in changes in levels of DJ-1 and CB_2 proteins in the hippocampus of diabetic rats[4,6,13]. The results of the present study showed that endurance training resulted in a non-significant decrease in DJ-1 protein content in the E group. This non-significant change could be attributed to an insufficient duration of the intervention period, and low duration and intensity of training sessions, or both.

In addition, the findings of the current study showed a strong correlation between the levels of DJ-1 and CB_2 with blood glucose. The level of glucose in the DE group was significantly lower than in the D group, suggesting that the prescribed exercise regimen resulted in a significant reduction of blood glucose after inducing diabetes. This reduction in blood glucose was accompanied by a decrease of DJ-1 and an increase of CB_2 in the hippocampus. If an increase in DJ-1 and a decrease in CB_2 levels in response to diabetes was associated with neurodegenerative disease, then the inclusion of regular exercise could reduce the blood glucose and lower the risk of developing neurodegenerative diseases. Previous studies suggested that the reduction of blood glucose during exercise and post-exercise might have beneficial effects on neuronal protective processes including anti-oxidative and anti-inflammatory systems, in addition, insulin sensitivity increases with exercise[29,51,53]. It is possible that exercise could affect the levels of DJ-1 and CB_2, and consequently the neurodegeneration process, by a reduction of the blood glucose concentration. It is shown that insulin treatment could improve the function of sensory neurons in diabetic specimens with developed neuronal disorders[54–56]. Although the relationship between an increase in blood glucose with neurodegeneration was not assessed in the current study, the strong positive relationship between DJ-1 and CB_2 with glucose could provide a valuable insight into a possible mechanism for the prevention of diabetes-induced neurodegeneration. Previous studies examined the role of DJ-1 and CB_2 in the function of neural cells and neurodegenerative diseases, and suggested that these proteins play roles in regulating free electrons, inflammatory factors and apoptosis[12,36]. In addition, studies have shown that nerve cells in diabetic rats might have mitochondrial disorders, increased inflammatory factors and oxidative stress[57,58].

In addition, studies have shown beneficial effects of exercise, such as improvement of the anti-oxidant system, increased mitochondrial biogenesis, reduction of inflammatory factors and reduced blood glucose[53]. Thus, changes in levels of DJ-1 and CB_2 proteins due to endurance activity might be the result of improving the anti-oxidant defense system, producing fewer free electrons, reducing inflammatory cytokines or reducing blood glucose. Considering the strong correlation between levels of DJ-1 and CB_2 proteins with blood glucose levels, the effects of these proteins on neuronal protection in diabetes patients might be associated with blood glucose levels. However, it should be noted that this hypothesis was not explored in the present study, and more studies are required to investigate the effect of DJ-1 and CB_2 on the neural health of diabetes patients.

An increase in blood glucose above normal levels could have significant effects on hippocampus DJ-1 and CB_2. However, inclusion of regular physical activity might mediate the effect of diabetes on DJ-1 and CB_2 by reducing blood glucose level.

DISCLOSURE

The authors declare no conflict of interest.

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