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Original Article

Effect of 6-week Endurance Training on the Hippocampal Tissue in the Streptozotocin-Induced Diabetic Rats: A Histological Study

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ABSTRACT

Background: Glial cells perform several critical functions and exercise can moderate many of these interventions, so a comprehensive study is needed to elucidate the mechanisms that influence brain function following diabetes and exercise. In this study, the quantitative histological changes of glial in the hippocampus of Streptozotocin (STZ)-induced diabetic rats following endurance training were investigated.

Methods: Twenty-four adult male Wistar Rats (aged 10 weeks with an average weight of 256 ± 11.8 g), were classified into four groups: Diabetic, diabetic trained, untreated control, and trained groups. Diabetes was induced by a single dose intraperitoneal injection of Streptozotocin (45 mg/kg). Then, moderate exercise was performed for 6 weeks (5 sessions in a week). Rats were anesthetized 48 hours after the last training session. Then, they were sacrificed and the hippocampal tissue was removed. Sections (5-6 µ) were prepared and stained with Hematoxylin and Eosin (H & E) staining method.

Results: Histological evaluations showed that the number of astrocyte and oligodendrocyte cells in different hippocampal regions in diabetic rats significantly decreased compared to the untreated control and trained rats. However, the number of microglia cells in diabetic rats was significantly higher than that in the untreated control and trained rats ($P < 0.05$). In addition, the number of astrocytes and oligodendrocytes significantly increased in the dentate gyrus, Cornu Ammonis, and subiculum of the hippocampal tissue after endurance training compared to the control group, while the number of microglial cells significantly decreased ($P < 0.05$).

Conclusion: The findings of the present study confirmed the potential effects of moderate exercise on diabetes. Therefore, it seems that physical activity plays an essential role in improving the nervous complications in patients with diabetes.

Keywords: Endurance training, Glial cells, Histological, Hippocampus, Diabetes

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Introduction

Diabetes is known as a chronic metabolic disorder divided into two main forms (type 1 and 2) with a significant index of hyperglycemia (1, 2). Diabetes has serious complications such as nephropathy, neuropathy, retinopathy, cardiovascular problems, gastrointestinal disorders, immune system disorders, and skeletal muscle atrophy (3). Another organ that is affected by diabetes is the central nervous system (CNS) (4). Hyperglycemia-induced oxidative stress has been implicated in the development of diabetic neuropathy, one of the complications of diabetes (1). Cellular mechanisms that describe how diabetes mellitus negatively affects the structure and function of the CNS are still not well known.

In recent years, numerous studies have shown that diabetes has a negative effect on the CNS in animal models (5). Neuropsychological researches have shown that patients with diabetes mellitus experience cognitive functions disorders in processes such as nonverbal memory and explicit verbal, attention, intellectual development, and processing speed (6). Research has shown that, under advanced inflammatory conditions due to hyperglycemia, the activity of microglia has increased and led to astrocytes hemichannels activity due to the release of pro-inflammatory cytokines. The activation of such hemichannels is the first step in the onset of neurotoxic intracellular cascades and the progression of cell death in astrocytes, neurons, and oligodendrocytes (7, 8). There is still no effective prevention for neurodegenerative disorders despite extensive research and ongoing laboratory efforts (9). Considering that the therapeutic methods used to treat the process of neurodegeneration caused by diabetes have little or no effect, or has symptoms such as acidosis and indigestion (10), therefore, researchers are looking for alternative ways to prevent or treat the process of neurodegeneration with fewer complications in diabetic patients.

Recently, strong evidence has shown that exercise contributes to the promotion of learning and memory, the delay in cognitive decline associated with age, decreases inflammatory factors or neurodegenerative disorder (11). Also, the previous study evidence has shown that physical activity, in addition to promoting behavioral performance, improves synaptic plasticity in the hippocampus (12). It has been confirmed that endurance training in diabetic rats decreased the activity of microglia cells, and subsequently, reduced the levels of inflammatory cytokines in the hippocampus (13). In addition, the proliferation of astrocytes after endurance training was associated with angiogenesis in the cortex of healthy mice. This event will protect the blood-brain barrier (BBB) following brain damage (14). Studies have shown that neural adaptation such as decrease of inflammation and oxidative stress following endurance training is crucial for the prevention and treatment of neurodegenerative diseases such as Alzheimer's and Parkinson's disease, and diabetic neuropathy (15). However, the possible mechanisms for the effect of physical activity on neurodegenerative disorders caused by diabetes are not well understood (16). Since the hippocampal region, which includes the Cornu Ammonis (CA), Dentate Gyrus (DG), and the subiculum (S) regions, is one of the critical areas of the CNS with a various function such as learning and memory, and also, is very sensitive to changes in glucose homeostasis (17), this study aimed to investigate the effect of endurance training on the histology of the hippocampal region in the experimental diabetic rats.

Materials and methods

Animals

In the present study, 24 adult male Wistar rats (aged 10 weeks, with an average weight of 256 ± 11.8 g) were kept in Plexiglas cages under standard conditions. Five animals were housed in each cage in a controlled room (12/12 h light-dark cycle) with free access to food and water. The rats were adapted with laboratory conditions, treadmill, and manipulation for 2 weeks (18). During the acquaintance stage, the rats run on the treadmill with a speed of 15 m/min for 15 minutes (5 days per week). The research protocol was approved by the Ethics Committee of Lorestan University of Medical Sciences (Ethical code: LU. ECRA. 2017.2).

Induction of diabetes

After 12 hours of fasting, diabetes was induced by intraperitoneal injection of 45 mg/kg of STZ [(Sigma, St. Louis MO, USA) STZ was prepared freshly by dissolving it in 0.5 M citrate buffer with pH 4.5)]. Non-diabetic rats were injected with the same volume of citrate buffer. After 48 hours, a drop of blood was placed on the glucometer tape by causing a small lesion on the venous tail vein, and blood glucose was

measured by a glucometer (Roche diagnostic, Japan). In this study, the rats with a blood glucose level above 300 mg/dl were considered as diabetic rats (19).

Grouping and Exercise Program

After the acquaintance stage, rats were randomly divided into four groups as follows: 1) Diabetic trained group (n=6), in which diabetes was induced by intraperitoneal injection of Streptozotocin (STZ) and they completed 5 sessions of endurance training or 6 weeks. 2) Diabetic group (n=6), which did not do exercise at any time. 3) Trained group (n=6), which similar to the diabetic trained group, participated in the treadmill exercise program. 4) Untreated control group (n=6), which were not engaged in any activity.

Endurance Training protocol

Animals in the training groups performed exercise protocol 5 days a week, for 6 weeks (Table 1). Before and after exercise training, 3-min warm-up and cool-down were carried out, respectively, and the treadmill slope was zero at all stages. Speed and time duration were kept constant during the final week (sixth week), to conserve the adaptations resulted from the 6-week endurance training (20). All training sessions were conducted between 08:00-12:00 AM.

Table 1. Endurance training protocol in different weeks

Weeks	First Week	Second Week	Third Week	Forth Week	Fifth Week	Sixth Week
Duration (min)	10	20	20	30	30	30
Speed (m/min)	10	10	15	15	17-18	17-18
Slope	0	0	0	0	0	0

Behavioral measurements

Object recognition test

In the object recognition test, each rat was placed in a white wooden box with dimensions of 40 × 40 × 16 cm for 15 minutes. After one week, two identical objects were placed in the box and each rat was housed in the box for 10 minutes. After 30 minutes, one of the objects was replaced to another color-differentiated object, and each rat was kept in the box for 5 minutes, while the whole time was captured with a camera. When reviewing the films, the rat was considered as favorable and indifferent as to the first move toward the new object, or the old object was considered as undesirable (21).

Tissue extraction and histological staining

After six weeks of training, the animals were anesthetized with ketamine (75 mg/kg) and xylazine (5 mg/kg), and after decapitation by guillotine, the hippocampal tissue was removed under sterile conditions and placed in 10% formalin solution. After 24 hours, the hippocampal tissue was removed and placed in 10% fresh formalin solution. Then, the hippocampal tissue was dehydrated with ethanol (70% for 24 hours, 90% for 1 hour, and 100% for 1 hour), and then, cleared with xylenol and placed in paraffin. In the next step, the samples were cut by a microtome (Leica RM2025, Germany) with a thickness of 5 microns, and then, fixed onto the glass slide after the usual stages of tissue consolidation. In the end, the glass slides were stained with the usual Hematoxylin-Eosin method (H & E) (22).

Histopathological assessment of the hippocampus

In this study, histological changes and quantity of glia cells in different parts of the hippocampus of the CNS were examined. Using microscope, the number of astrocytes, oligodendrocytes, and microglia at X40 magnification (5 microscopic slides for each sample) in CA1, CA2, CA3, CA4, dentate gyrus, and subiculum areas was measured and counted. At this stage, the DinoCapture software and the Dino-Eye Microscope AM-423X (ANMO, Taiwan) were used. In H & E staining, astrocyte cells can be detected concerning their oval nucleus (8). These cells have the largest nucleus among glia cells. Also, in the tissue section, the oligodendrocytes have small spherical and dark nuclei (heterochromatin). In the H & E staining, microglia can be detected by small nuclei and their heterochromatin (8). Using the Atlas of the CNS, different hippocampal regions were identified in the cross-section, and the measurement and counting operations were performed (23).

Statistical analysis of data

Normality of data distribution and homogeneity of variances were assessed using Kolmogorov-Smirnov and Leven's tests, respectively. Mixed ANOVA was used to

evaluate glucose changes from 48 hours after STZ injection to the end of training. One-way ANOVA was used to compare changes in the number of cells between different groups. The Tukey's post-hoc test was used to analyze the variance for the paired comparison. All data related to table and diagrams were presented as the mean ± standard error of the mean (SEM). Statistical significant level was considered at $P < 0.05$. The statistical analysis was performed using SPSS software version 18.

Results

Blood glucose level

At the beginning of the exercise program, 48 hours after induction of diabetes, blood glucose levels increased significantly in the diabetic rats ($P < 0.001$), and after 6 weeks of endurance training compared to the untreated control and trained groups, it was also significantly different ($P < 0.001$). At the end of the last session of training, the blood glucose concentration in the diabetic trained group was significantly lower than the untreated control group of diabetes ($P < 0.001$) (Figure 1).

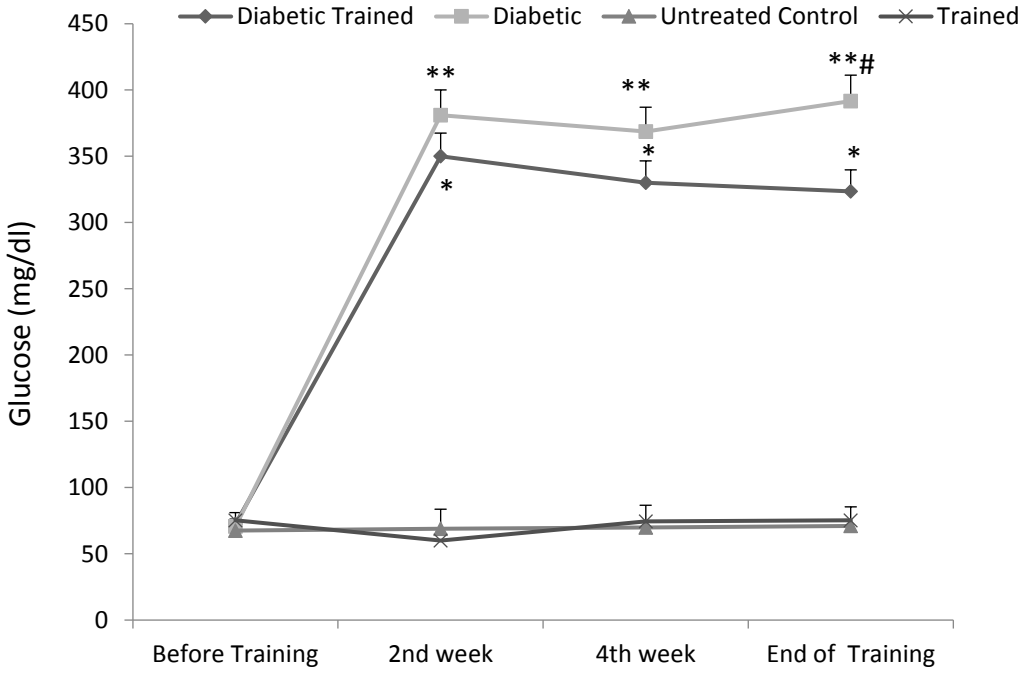


Figure 1. Changes in the serum levels of glucose during 6 weeks of endurance training.
*; Significant difference ($P < 0.001$) between diabetic trained group in the 2nd week, 4th week, and end of training with untreated control and trained groups. **; Significant difference ($P < 0.001$) between diabetic group in the 2nd week, 4th week, and end of training with untreated control and trained groups. #; Significant difference ($P < 0.001$) between diabetic trained and diabetic groups at the end of training. Data are expressed as mean ± SEM.

Object recognition test

Figure 2 shows the results of the object recognition test. According to these figures, at the end of the sixth week of training, cognitive function was significantly improved in the diabetic group compared to the untreated control group ($P < 0.05$). In the untreated control and trained groups, exercise also improved cognitive function.

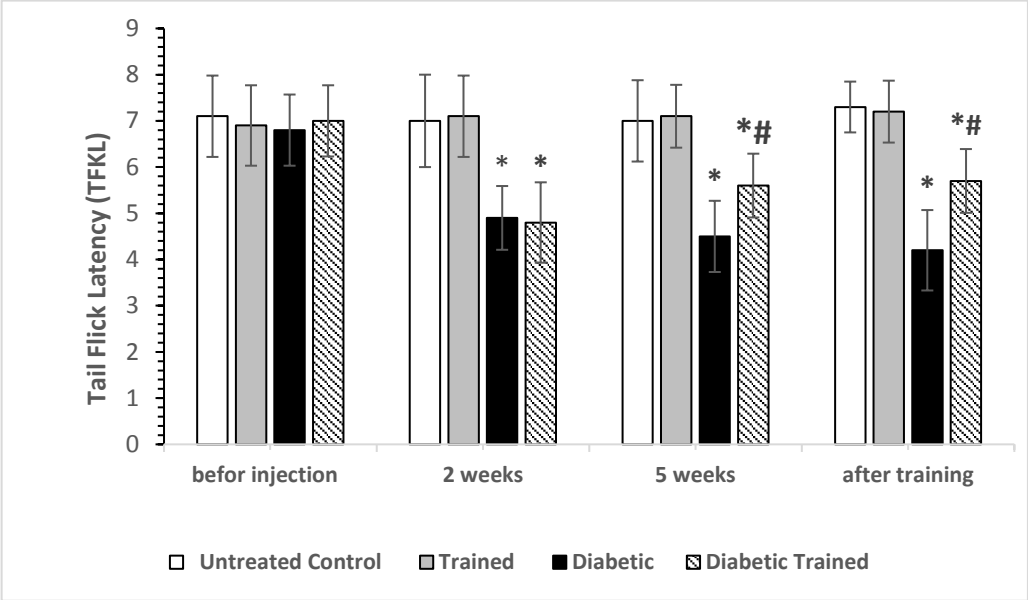


Figure 2. Thermal hyperalgesia in the experimental groups before STZ injection, and two, five, and six weeks after diabetes induction.
* Significant difference ($P < 0.05$) between diabetic trained and diabetic groups in the 2nd week, 5th week, and after training with untreated control and trained groups. # Significant difference ($P < 0.05$) between diabetic trained and diabetic groups in the 5th week and after training. Data are expressed as mean ± SEM.

Histopathological results of the hippocampus

In Figure 3, the histological examination of hematoxylin and eosin-stained sections of the hippocampus in rats is shown. In this figure, different regions of the hippocampus are shown.

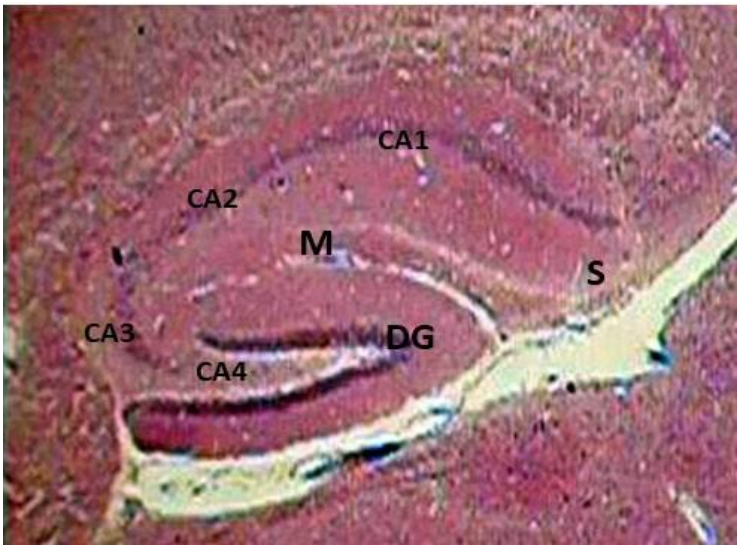


Figure 3. Section from different hippocampal regions that formed of the Cornu Ammonis (CA) as CA1, CA2, CA3, and CA4 regions, and is followed as subiculum (S). Dentate gyrus (DG) is seen encircling CA4 by its upper and lower limbs (H & E X4).

As shown in Table 2, the analysis of variance among different groups in two layers of hippocampus, including pyramidal and molecular, showed a significant difference between the number of astrocytes, oligodendrocytes, and microglia in both pyramidal and molecular layers of different regions of the hippocampus ($P < 0.05$).

Table 2. Analysis of variance between different groups in two layers of the hippocampus including pyramidal and molecular ($P < 0.05$)

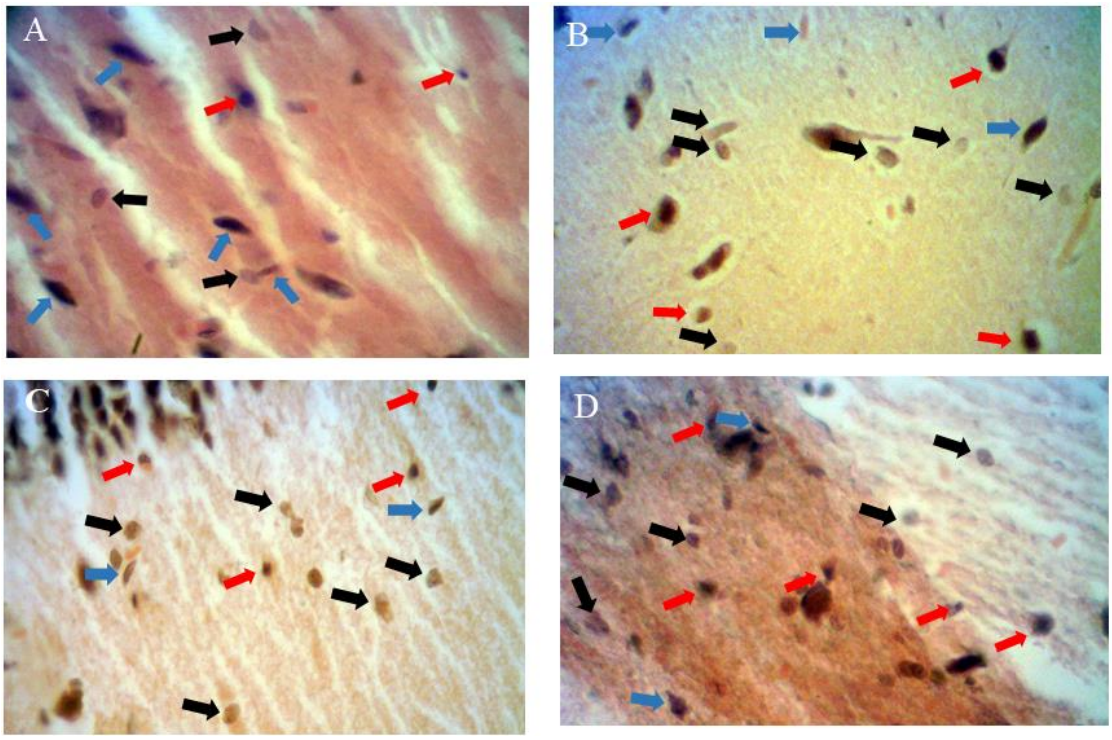
	Pyramidal Layer						Molecular Layer					
Regions	DG	S	CA1	CA2	CA3	CA4	DG	S	CA1	CA2	CA3	CA4
Cell Type												
Astrocyte	0.001	0.004	0.034	0.001	0.013	0.043	0.001	0.023	0.002	0.022	0.041	0.017
Oligodendrocyte	0.002	0.013	0.001	0.022	0.002	0.001	0.001	0.017	0.046	0.034	0.025	0.043
Microglia	0.011	0.001	0.001	0.001	0.029	0.001	0.001	0.041	0.022	0.001	0.007	0.09

The results of the post-hoc test showed that the number of astrocytes and oligodendrocytes in different regions of the hippocampus in diabetic rats, both in the pyramidal and molecular layers, was significantly lower than that in the untreated control and trained rats ($P < 0.05$). However, the number of microglia cells in the pyramidal and molecular layers in diabetic rats was significantly higher than that in the untreated control and trained rats ($P < 0.05$) (Table 3). Also, after the end of 6 weeks of training, the results of microscopic observation showed that the mean number of astrocyte and oligodendrocyte cells in the pyramidal and molecular layers of the different regions of the hippocampus in the diabetic trained group did not show a significant difference compared to the untreated control group ($P > 0.05$). However, the results proved that the number of microglia cells in the pyramidal and molecular layers in the diabetic trained rats was significantly lower than that in the diabetic rats ($P < 0.05$). In Figures 4-6, changes in the cells of different regions of the cross-section of the hippocampal tissue in four groups are schematically shown.

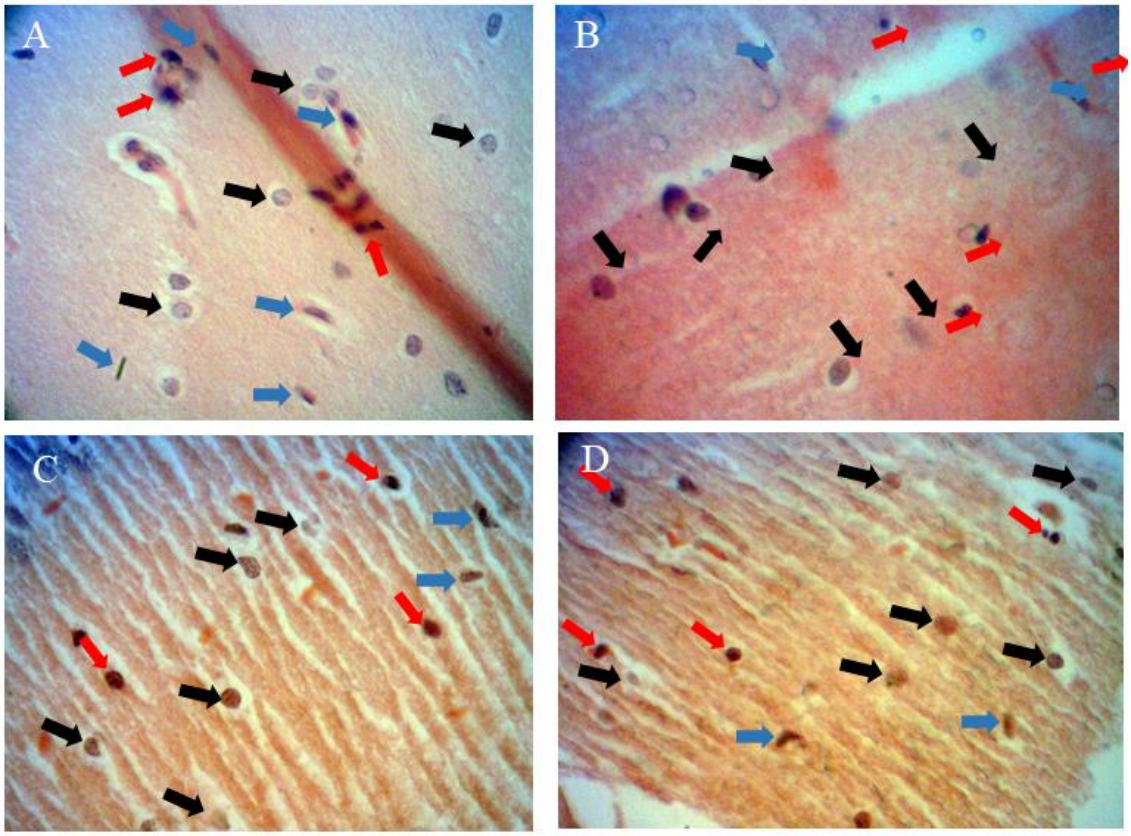
Table 3. Mean ± SEM of one-way ANOVA with Tukey's post-hoc test of the number of astrocytes, oligodendrocytes, and microglia in different layers

Region	Layer	Cell	Diabetic	Diabetic Trained	Untreated Control	Trained
Dentate gyrus	**Pyramidal**	Astrocyte	15.6±1.5*	27.4±4	22.3±1	25.7±1.2
		Oligodendrocyte	17.8±0.7	19.9±3.5	10.5±1.6**	17.6±2.8
		Microglia	2.8±0.6	2.3±.5	3.2±2.7	4.2±.5
	Molecular	Astrocyte	24.5±1.5*	28.9±1.1	19.1±2.3	25.9±1.7
		Oligodendrocyte	11.8±1.8 *	25.2±2.7ǂ	16.3±.8	18.2±1.5
		Microglia	5.5±.6*	2.6±.6	2.3±1.6	2.6±.3
Cornu Ammonis CA1-CA4	**Pyramidal**	Astrocyte	10.2±2.9*	14.9±3.5	16.3±.4	20.7±4.1#
		Oligodendrocyte	7.8±2.2*	16.9±2.4	12.5±1.9**	16.6±1.3
		Microglia	8.8±1.6*	2.8±.8	4.3±2.7	3.2±1.1
	Molecular	Astrocyte	11.5±3.5	13.9±2.1	15.5±2.8	21.4±5.3#
		Oligodendrocyte	9.8±2.4*	14.2±3.7	16.3±.8	20.2±4.5#
		Microglia	9.5±3.7*	3.6±1	4.3±1.8	2.9±1.2
Subiculum		Astrocyte	13.1±2.5*	16.1±1.22	17.2±1.21	19.6±5.3
		Oligodendrocyte	11.8±.7*	15.9±3.5ǂ	18.5±1.3	20.6±2.1
		Microglia	6.4±1.9*	2.9±1.5	3.2±2.1	4.4±.5

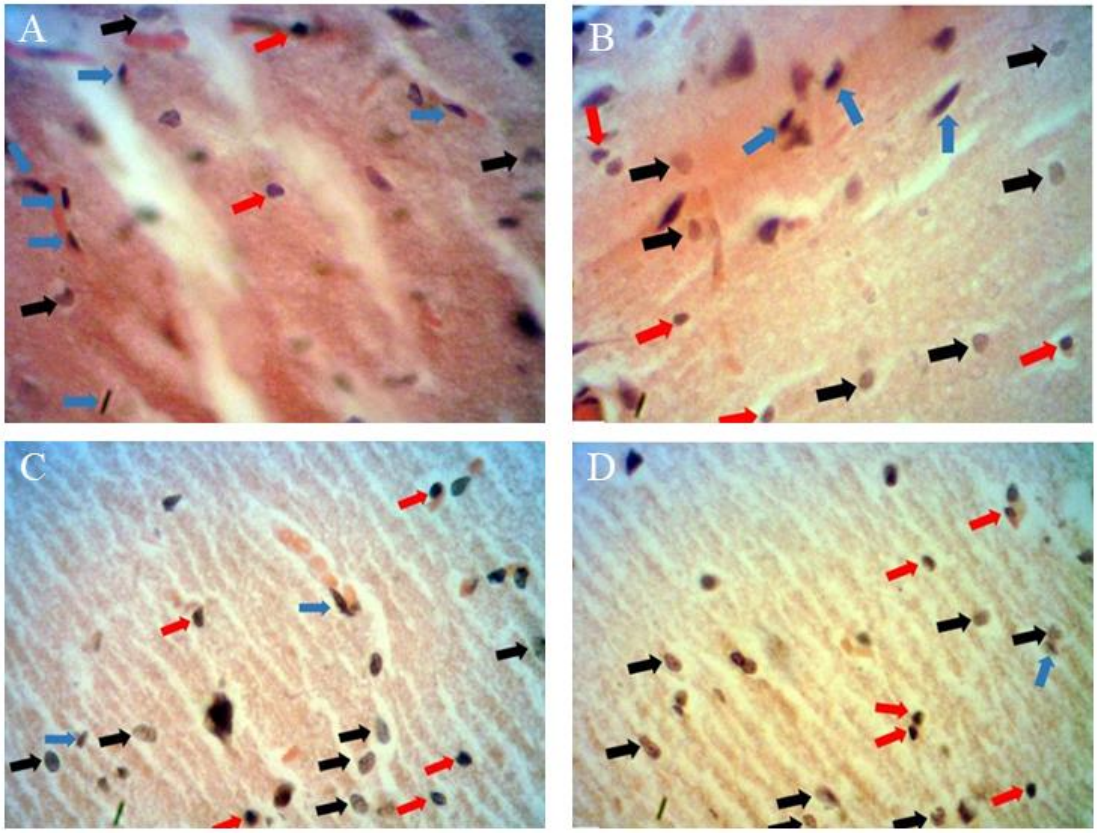
*Significant difference ($P < 0.05$) between diabetic group with another groups. ** Significant difference ($P < 0.05$) between untreated control group with another groups. ǂSignificant difference ($P < 0.05$) between diabetic trained group with another groups. # Significant difference ($P < 0.05$) between trained group with another groups. Data are expressed as mean ± SEM.



Figures 4. Selective histological sections of pyramidal and molecular layers of the dentate gyrus (DG) regions of the hippocampal tissue. A) Diabetic; B) Diabetic trained; C) Untreated control; D) Training. (Black arrows show astrocyte cells, red arrows show oligodendrocyte cells, and blue arrows show microglia cells) (H & E X40).



Figures 5. Selective histological sections of pyramidal and molecular layers of the Cornu Ammonis (CA) regions of the hippocampal tissue. A) Diabetic; B) Diabetic trained; C) Untreated control; D) Trained. (Black arrows show astrocyte cells, red arrows show oligodendrocyte cells, and blue arrows show microglia cells) (H & E X40).



Figures 6. Selective histological sections of pyramidal and molecular layers of the subiculum (S) regions of the hippocampal tissue. A) Diabetic; B) Diabetic trained; C) Untreated control; D) Trained. (Black arrows show astrocyte cells, red arrows show oligodendrocyte cells, and blue arrows show microglia cells) (H & E X40).

Discussion

Diabetic hyperglycemia causes complications such as DNA damage, chronic oxidative stress, and neuronal death (24). Increased glial response is a common symptom of diabetes mellitus (25). Therefore, in the present study, the effects of 6 weeks of endurance training against hyperglycemia and oxidative stress and glial reactions were studied. The results of this study showed that 6 weeks of moderate training significantly reduced blood glucose concentration in diabetic trained group. These results are consistent with the results of previous research showing that endurance training increases the expression of glucose transporters and can reduce blood glucose levels in animals and humans with diabetes (26, 27). Endurance training can help reduce plasma glucose levels during and after exercise, and it has been shown that exercise can also increase insulin sensitivity (28). In addition, the results indicate that endurance training increased cognition of the new object, and thus, preventing object recognition disorders in diabetic rats with streptozotocin. Recent research has shown that hyperglycemia and oxidative damage in the hippocampus has been reduced during endurance training with sub-maximal severity, and regular exercise can promote memory performance with an anti-oxidative effect (29).

Astrocyte cells are considered as energy storage sources by storing glycogen and releasing glucose (8). The oligodendrocytes in the white matter, with the formation of the myelin sheath around the axons, accelerate the transfer of action potentials in the CNS (8). The present study confirmed that the number of astrocyte and oligodendrocyte cells of the hippocampus in diabetic rats decreased compared to the untreated control group. While, 6 weeks of endurance training on the treadmill prevented the decrease in the number of astrocyte and oligodendrocyte cells and improved the number of these cells, which is consistent with the results of the research by de Senna et al. (2011) (8). Endurance training extends indices such as neurogenesis (30), long-term potentiation, synaptic plasticity (31), and increased the expression of neurotrophic factor (32), and prevents oxidative stress (33). In contrast, hyperglycemia associated with diabetes leads to a substantial decrease in hippocampal proliferation (34) and increased oxidative stress and neuronal death (35). Microglia explained an

essential population of macrophages-like cells in the CNS considered immune sentinels capable of orchestrating a potent inflammatory response (36). Microglia are also involved in synaptic organization, phagocytosis of apoptotic cells in the developing brain, control of neuronal excitability, myelin turnover, phagocytic debris removal, as well as brain protection and instauration (37). Coordinated interaction between cells is essential to develop the extremely and dynamic functions performed by the CNS (37). Orellana et al. (2013) reported that during inflammatory processes in neurodegenerative diseases, opening of the hemichannels reduces neuronal immunity (38). Increased activity of astrocytes and neuronal hemi channels leads to the release of neurotoxic molecules such as glutamate and ATP, which can release more cytokines in microglia and leading to cell death and degradation of CNS function, as seen in diabetes mellitus and Alzheimer's disease (38, 39). This study showed that endurance training reduces microglial cells in the hippocampal tissue, in both pyramidal and molecular layers. Yoo et al. (2015) have also shown that endurance training on the treadmill significantly reduced microglial cells and moderated the increase in inflammatory factors such as TNF-α, IL-6, and IL-1β induced by diabetes (13). Also, de Senna et al. (2011) showed that the levels of glial cells of the hippocampal CA region were increased in diabetic animals following endurance training on the treadmill (8). They reported that since astrocytic proliferation after endurance training has already been demonstrated in other brain areas in the untreated control, trained and diabetic rats, their results are probably not special to the CA1, and changes in factors such as BDNF and IGF could not be ignored (8). Therefore, endurance training may preserve the blood-brain barrier (BBB) from damage caused by diabetes or other types of brain damage (8, 40). Investigating the factors affecting the process of neurodegeneration in diabetes requires more extensive research in the future. In addition, the effects of different types of physical activity on the prevention of diabetes complications on the central nervous system may be the subject of future research.

Conclusion

According to the results of the present study, considerable changes occur in the histology of the hippocampus of diabetic rats following the development of exercise programs, which can indicate the beneficial effect of the basic endurance training on the CNS homeostasis. The results of this study showed that endurance training reduces microglia cells, and thus, moderated the increase in inflammatory factors. Therefore, by conducting supplementary studies and possible confirmation, these results can prevent and treat disorders caused by nerve degradation in diabetic patients.

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Conflict of interests

The authors declare that they have no conflict of interests.

Author's contributions

Rami, M., participated in study design, histology evaluation, animal training, data collection and evaluation, drafting and statistical analysis. Fathi, M., contributed extensively to the interpretation of the data and the conclusion. Rahmati, M., conducted molecular experiments and data analysis. Tabandeh M R., participated in the study design, data collection and evaluation, drafting and statistical analysis. All authors performed editing and approving the final version of this paper for submission, and participated in the finalization of the manuscript and approved the final draft.

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