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Protective Effect of Intranasal Insulin Administration on Cognitive Functions and Neurogenesis in a Rat Model of Alzheimer's Disease

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ABSTRACT

Background and Objective: Alzheimer's disease is the most common destructive brain disease which is associated with cognitive disorders. Considering the protective role of insulin in the functions of the nervous system, the present study was conducted to investigate the effect of intranasal insulin administration on cognitive disorders and neurogenesis in rats treated with streptozotocin (STZ).

Methods: In this experimental study, 32 male Wistar rats were divided into 4 groups of 8: control, STZ, STZ + insulin and insulin. The model of Alzheimer's disease was induced by intraventricular injection of STZ (3 mg/kg; 3 µl in each ventricle). Two weeks after STZ injection, cognitive functions were evaluated using Elevated Plus Maze (EPM) and Passive Avoidance (PA) tests. Insulin treatment (2 IU daily; 10 µl in each nasal passage) was performed after STZ injection for 14 consecutive days. The change in the expression of genes involved in neurogenesis (Nestin, DCX and Ki67) in the hippocampus area was investigated by Real-time PCR technique.

Findings: STZ caused longer animal stay in open arms in acquisition phase (64.5±5.24) and recall phase (60.25±5.55) compared to the control group (33±2.17 and 26.38±2.06) in the EPM test ($p<0.05$ and $p<0.01$, respectively). In addition, it caused a decrease in learning recall 90 minutes (77.57±6.03) and 24 hours (90.25±7.25) after training, compared to the control group (254.38±3.19 and 238.13±3.46) in the PA test ($p<0.001$ and $p<0.05$, respectively). Insulin treatment improved the above parameters in EPM test (41.88±4.14 and 31.5±4.16, respectively) and PA (278.88±2.32 and 218.5±2.12, respectively) compared to the STZ group. STZ also led to a decrease in Nestin gene expression (0.46±0.04), DCX (0.35±0.04) and Ki67 (0.41±0.05) compared to the control group (1.02±0.11, 1±0.04 and 1.01±0.08, respectively) ($p<0.01$, $p<0.001$ and $p<0.001$, respectively), while insulin treatment could increase the expression of these genes (0.87±0.09, 0.78±0.02 and 0.69±0.08, respectively) ($p<0.05$).

Conclusion: The results showed that insulin improved cognitive functions and increased neurogenesis in rats treated with STZ. Therefore, insulin can be considered as an effective therapeutic target in Alzheimer's disease.

Keywords: *Streptozotocin, Alzheimer's Disease, Insulin, Neurogenesis.*

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Introduction

Alzheimer's disease is a neurodegenerative disease that is associated with progressive disorders in cognitive functions. Loss of memory, lack of attention and concentration, impaired thinking and reasoning, mood and behavioral changes such as confusion, anxiety and depression are some of the obvious symptoms of this disease that can disrupt a person's ability to perform daily activities. Accumulation of amyloid plaques, oxidative stress, neuroinflammation and mitochondrial dysfunction are among the most important pathophysiological features in the brain of patients with Alzheimer's disease, which can eventually lead to cognitive disorders (1).

Intraventricular injection of Streptozotocin (STZ) to rats, in doses lower than the diabetes-causing dose (3 mg/kg), is one of the suitable methods for inducing Alzheimer's disease in the shortest possible time (two weeks after injection) (2, 3). In this method, STZ disrupts the absorption of brain glucose, causes oxidative stress, causes inflammation, accumulates hyperphosphorylated tau, disrupts the insulin signaling pathway, reduces the activity of the acetylcholine transferase enzyme, and damages cholinergic neurons, causing memory and learning impairment (3, 4). Considering that the intraventricular injection of STZ causes behavioral, molecular and pathological changes similar to Alzheimer's disease, this method can provide a suitable animal model for the induction of Alzheimer's disease and the study of molecular signaling destroyed in this disease (2).

Investigating the changes in the neurogenesis process in the brain of patients with Alzheimer's disease has attracted a lot of attention. Accordingly, some studies have reported an increase in neurogenesis (5, 6) and others a decrease in neurogenesis (7, 8) in Alzheimer's disease. Neurogenesis, in a comprehensive definition, is the process of producing functional neurons from neural progenitor cells, which is usually observed in mammals in embryonic and prenatal stages, and in the brain of adult mammals, this process is limited to certain areas of the brain such as the subgranular zone and the subventricular zone, and it is necessary to maintain synaptic plasticity and memory formation (9). Studies have shown that neurotrophic factors and growth factors are important regulators of the neurogenesis process in the adult brain, among which insulin can be mentioned (10).

Insulin has many protective effects in the functions of the central nervous system; reduced levels of insulin or disturbance in the signaling pathway of insulin have been reported in neurodegenerative diseases such as Alzheimer's disease (11). However, studies have shown that intranasal administration of insulin improves memory in rat models of Alzheimer's disease by affecting the areas involved in cognitive processes (12, 13). In this method of administration, insulin easily enters different areas of the brain such as the hippocampus and cortex, and unlike other injection methods such as intravenous or subcutaneous injection, which are invasive methods and come with side effects such as hypoglycemia, the amount of insulin and peripheral blood glucose remain unchanged in intranasal administration of insulin (14).

Although studies have reported the positive effect of insulin in improving the memory of Alzheimer's patients or animal models of this disease, the underlying molecular and cellular mechanisms of this disease are not fully understood. Considering the importance of neurogenesis in cognitive processes and the existence of conflicting studies related to the changes of neurogenesis in the brains of patients with Alzheimer's disease, the purpose of this study is to investigate the effect of intranasal administration of insulin on memory acquisition and recall using the Elevated Plus Maze (EPM) test, the evaluation of learning recall using the passive avoidance test and also the evaluation of the changes in the expression of important genes involved in the neurogenesis process in STZ-treated rats to determine whether intranasal

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administration of insulin, based on the protocol presented in this research, can improve or reduce cognitive deficits in an animal model of Alzheimer's disease by modulating the expression of factors involved in neurogenesis.

Methods

This experimental study was carried out on 32 male Wistar rats (200-250 grams) after approval by the ethics committee of Kharazmi University with code IR.KHU.REC.1401.026. The animals were kept in standard cages in standard conditions of temperature (22±2°C), humidity (50±10%) and light (12:12 light-dark cycle) with sufficient access to water and food. The rats were randomly divided into 4 groups of 8:

1. Control group: This group first received saline intraventricularly (3 µl in each ventricle) and then intranasally (10 µl in each nasal passage) for 14 days.

2. STZ group: This group received STZ (3 mg/kg) intraventricularly (3 µl in each ventricle) and then saline intranasally (10 µl in each nasal passage) for 14 days.

3. STZ and insulin treatment (STZ+Ins): This group received STZ (3 mg/kg) intraventricularly (3 µl in each ventricle) and then received insulin (2 IU daily) intranasally for 14 days (10 µl in each nasal passage).

4. Insulin group (Ins): This group received saline intraventricularly (3 µl in each ventricle) and then intranasally (10 µl in each nasal passage) insulin (2 IU daily) for 14 days. The protocol of this study is presented in Figure 1.

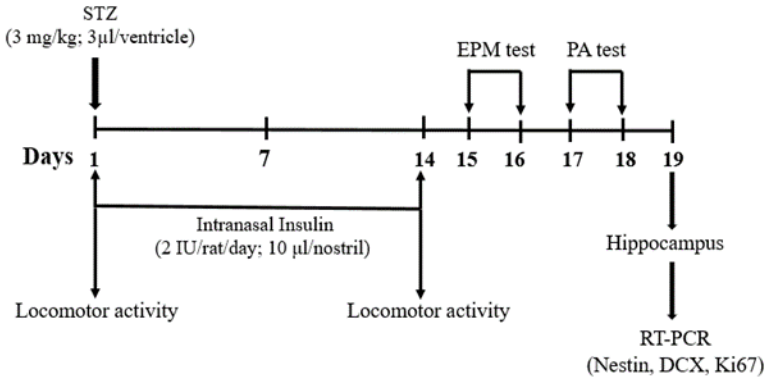

Figure 1. Scheduling protocol for drug administration, behavioral tests, and molecular studies

Induction of Alzheimer's disease: For this purpose, STZ purchased from Sigma Company (USA) was used. The selection of the dose of STZ (3 mg/kg) and its injection as a single dose on day 1 was based on previous studies (2, 3, 15). Animals were anesthetized with a combination of ketamine (80 mg/kg) and xylazine (20 mg/kg), and were placed in the Stereotaxic machine (Stoelting, USA). After determining the Bregma and Lambda points, the coordinates of the lateral ventricles for STZ injection were determined according to the Paxinos atlas (AP=-0.8 mm, ML=±1.5 mm and DV=-3.6 mm). Then, using a Hamilton microsyringe, 3 µl STZ (3 mg/kg) was injected at an injection rate of 1 µl per minute in each ventricle. Intranasal administration of insulin was done by micropipette for 14 consecutive days, according to the protocol (Figure 1). Each conscious animal received 20 µl of insulin daily, containing 2 units of insulin (2 IU), by intranasal administration (10 µl in each nasal passage) (13).

Assessment of motor activity: Open field device was used for this purpose. This device includes a box made of transparent compressed plastic at dimensions of 40x40x40 cm, which is equipped with infrared sensors at a distance of 2.5 cm from the bottom edge of the box. Before starting and after finishing the

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injection of drugs (days 1 and 14, respectively), the animal was placed in the device for 5 minutes, and then the animal's horizontal movement activities (the number of passes in front of the infrared sensors) were recorded by the device during this time and it was used as an index of motor activity (16).

Evaluation of memory acquisition and recall: For this purpose, Elevated Plus Maze (EPM) was used. This device has four arms, two of which do not have any walls and measure 50x10 cm. The other two arms have dark colored walls at dimensions of 40x10x50 cm. The height of this device is 50 cm from the ground and therefore it is called high maze. This test was done in 2 stages and on 2 consecutive days. On day 15, memory acquisition and 24 hours later, memory recall was examined. To evaluate memory acquisition, each animal was placed at the end of one of the open arms with its back to the center of the device, and then the time it took for the animal to enter from the open arm to one of the closed arms was considered as Initial Transfer Latency (ITL). In memory recall stage, the animal was placed at the end of the same arm of the game that was placed in the previous stage, and the time it took for the animal to enter from the open arm to one of the closed arms was considered as Retention Transfer Latency (RTL). The total time of this test was 90 seconds, and if the animal did not find the closed arm during this time, the RTL was recorded for the animal at 90 seconds. A decrease in RTL was considered as an indicator of improved cognitive function (17).

Assessment of passive avoidance memory: The device for measuring this test consists of two separate parts that are separated by a guillotine door. At the bottom of each section, there are 3 mm diameter rods that are spaced 1 cm apart. The dimensions of each section are 30x20x20 cm, and a 10-watt lamp is placed on top of the light chamber. This test was done in 3 phases and in 2 consecutive days:

1. Habituation phase: In this phase, each animal was placed inside the bright section with its back to the door. 10 seconds later, the door was removed to allow the animal to enter the dark section, as soon as it entered this section, the door was closed so that the animal could move freely there for 30 seconds.

2. Training phase: This phase was done 30 minutes after stage 1. First, the animal's hands, feet, and tail were wet, and then it was placed in the bright part with its back to the door. After 10 seconds, the door was removed and the animal was allowed to enter the dark area. As soon as the animal entered the dark area, the door was closed and an electric current with an intensity of 1 mA and a frequency of 50 Hz was passed through the animal's legs for 2 seconds. 20 seconds after the application of the shock, the door was removed to allow the animal to enter the light area. If the animal stays in this section for 120 seconds after entering the light section and does not enter the dark section, it indicates the formation of learning in the animal.

3. Learning recall phases: These phases were performed 90 minutes and 24 hours after the training step, with the difference that no electric shock was applied to the animal's feet. Step-through latency (STL) was recorded within 300 seconds (18).

Real-Time PCR (RT-PRC) technique: To measure the expression levels of genes Nestin (neural stem cell marker), Doublecortin (DCX) (neural stem cell to neuroblast differentiation marker) and Ki67 (neural stem cell proliferation marker) in the hippocampus, this technique was used. RNA extraction was done by a kit (Total RNA Extraction Kit, Pars Tous). The quantity and quality of extracted RNA were evaluated by Nanodrop device and 1% agarose gel, respectively. cDNA synthesis was performed using the instructions of the kit (Easy cDNA Synthesis Kit, Pars Tous). The reaction mixture was formed in a final volume of 15 µl, which included 2 µl of cDNA, 1 µl of forward and reverse primers, 7.5 µl of master mix containing SYBR Green and 3.5 µl of double-distilled distilled water. Reaction was performed using Applied Biosystems™ StepOne™ Real-Time PCR System and time and temperature plan of 95 °C for 15 minutes (1 cycle), repeating 40 cycles including: 95 °C for 30 seconds, 60 °C for 30 seconds and 72 °C for 30 seconds. The sequence of primers used in this study was designed with the help of Oligo analysis software and synthesized by TAKAPO TEB Company (Table 1). Data analysis was done using $2^{-\Delta\Delta Ct}$ formula. The

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results of all genes were normalized to the beta-actin gene, as an internal control, and then comparisons were made between groups (19).

Table 1. Sequence of primers used in this study

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
Nestin	GGAGCAGGAGAAGCAAGGTC	GAGTTCTCAGCCTCCAGCAG
DCX	GGAAGGGGAAAGCTATGTCTG	TTGCTGCTAGCCAAGGACTG
Ki67	CGGCGAGCCTCAAGAGATA	CGTGCTGTTCTACATGCCC
β-actin	TCTATCCTGGCCTCACTGTC	AACGCAGCTCAGTAACACTCC

Data analysis: Before performing statistical tests, Kolmogorov-Smirnov test was used to check the normality of data distribution. One-Way ANOVA was used to analyze the data obtained from EPM, PA and RT-PCR tests, and Tukey post hoc test was used to identify groups with significant differences. Repeated-measures ANOVA statistical test was also used to analyze the data obtained from the Open filed test. SPSS software version 22 (IBM, SPSS, Armonk, NY, USA) was used for statistical analysis and Graph Pad Prism software version 8 (Graph Pad Software, USA) was used to draw graphs and $p<0.05$ was considered significant.

Results

Examining the results regarding animal motor activity showed that there was no significant difference between the movement activity of the control group and the STZ group on day 1 (234.2±4.3 vs. 215.8±6.1) and day 14 (250.1±3.56 vs.195.2±5.21). Moreover, the results did not show a significant difference in the motor activity of the STZ group and the STZ group treated with insulin on day 1 and 14 (244.5±3.2 and 241.1±3.04, respectively). No significant difference was observed between the control group and the group receiving insulin alone on day 1 (195±2.4) and day 14 (221.2±3.4) ($p>0.05$) (Chart 1).

The results of the study also showed that the intraventricular injection of STZ significantly increased the presence of the animal in the open arms in the acquisition phase (64.5±5.24) and memory recall (60.25±5.55) compared to the control group (33±2.17 and 26.38±2.06) in EPM test ($p<0.05$ and $p<0.01$, respectively), which indicates the occurrence of disorder in acquisition and memory recall due to STZ injection. However, insulin treatment decreased the time spent in the open arms in the acquisition phase (41.88±4.14) and in memory recall (31.5±4.16) compared to the STZ group ($p<0.05$), but this reduction was not statistically significant in the memory acquisition phase. These results indicate an increase in the animal's ability to quickly find closed arms in the STZ group treated with insulin compared to the STZ group. Administering insulin alone did not show a change in acquisition (35.5±6.43) and memory recall (35±5.31) compared to the control group (Chart 2A and 2B).

Furthermore, the results of this study showed that STZ injection caused a significant decrease in step through latency (STL) in entering the dark area in the phase of learning recall, 90 minutes (77.57±6.03) and 24 hours (90.25±7.25) after training, compared to the control group (254.38±3.19 and 238.13±3.46) in the PA test ($p<0.001$ and $p<0.05$, respectively). However, insulin treatment increased STL in entering the dark area in these two phases (278.88±2.32 and 218.5±2.12, respectively) compared to the STZ group, which indicates an increase in animal's passive avoidance memory by insulin in shock-assisted recall in the dark part of the device. It is worth noting that insulin administration alone had no effect on these phases (278±4.53 and 215±5.11, respectively) compared to the control group (Charts 3A and 3B).

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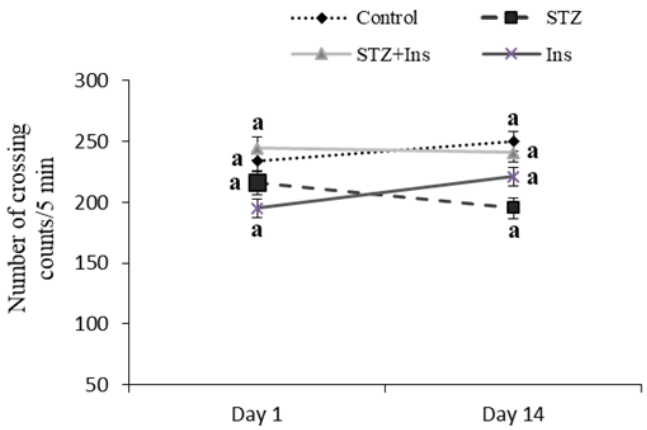

Chart 1. The effect of STZ and insulin injection on animal movement activity (letter a indicates no significant difference)

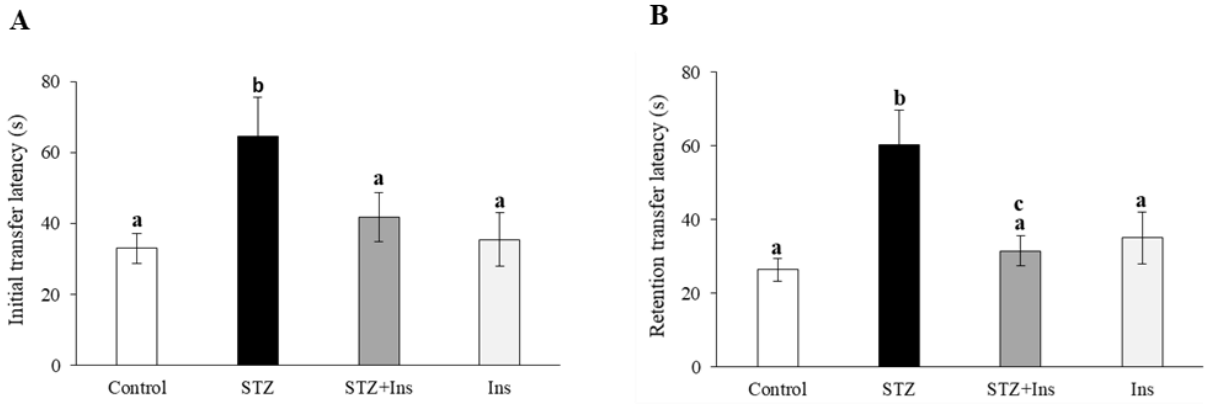

Chart 2. Effect of intranasal administration of insulin on memory acquisition and recall in STZ-treated rats in the EPM test. Diagram A: Memory acquisition, Diagram B: Memory recall. (a: no significant difference, b: difference compared to control group, c: difference compared to STZ group)

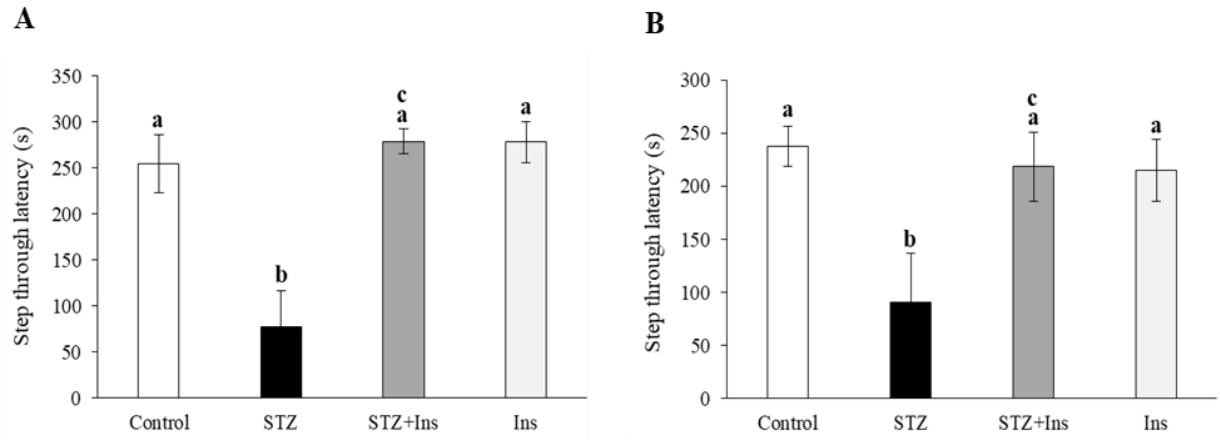

Chart 3. Effect of intranasal administration of insulin on learning recall in STZ-treated rats in PA test. Chart A: Learning recall 90 minutes after training, Chart B: Learning recall 24 hours after training (a: no significant difference, b: difference compared to the control group, c: difference compared to the STZ group)

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The results of molecular studies showed that in the STZ group, compared to the control group, Nestin (0.46±0.04 vs. 1.02±0.11), DCX (0.35±0.04 vs. 1±0.04) and Ki67 (0.41±0.05 vs. 1.01±0.08) gene expression decreased significantly ($p<0.01$, $p<0.001$ and $p<0.001$, respectively). However, insulin treatment caused a significant increase in Nestin (0.87±0.09), DCX (0.78±0.02) and Ki67 (0.69±0.08) gene expression compared to the STZ group ($p<0.05$, $p<0.001$ and $p<0.05$, respectively). Administration of insulin alone did not affect Nestin (0.91±0.04), DCX (0.92±0.05) and Ki67 (0.97±0.01) gene expression compared to the control group (Chart 4A, 4B and 4C).

In order to evaluate the effect of intraventricular injection of STZ and intranasal administration of insulin on animal weight, the weight of animals was measured on day 1 (before treatment), day 7, and also after the last intranasal administration of insulin (day 14) in all groups. The results did not show a significant change in the weight of animals of different groups. In addition, to ensure no changes happen in peripheral blood glucose and to avoid hypoglycemia, which may affect the animals' behavioral activities, blood glucose was measured 30-40 minutes after intranasal administration of insulin by taking blood from the tail of a conscious animal and using a glucometer device (it takes about 15-20 minutes for insulin to enter the brain in rats through the nose) (20). The results did not show a significant change in the amount of peripheral blood glucose in animals of different groups.

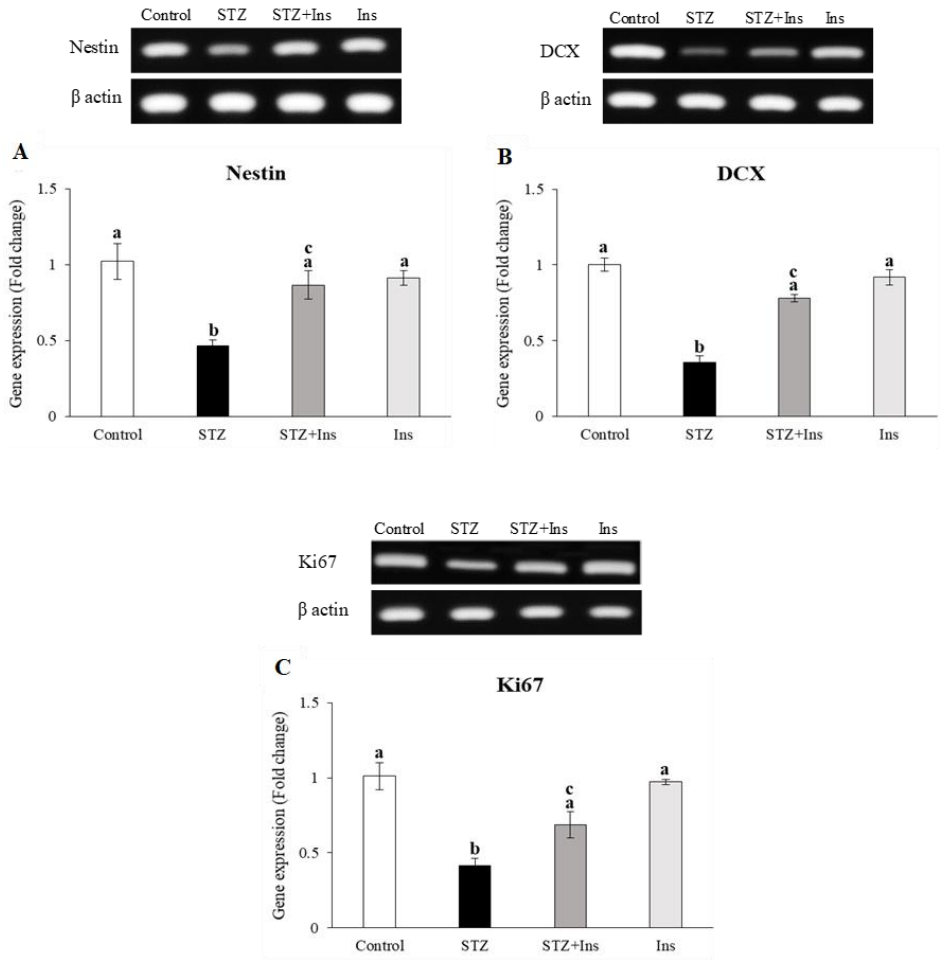

Chart 4. Effect of intranasal insulin administration on neurogenesis markers in STZ-treated rats. A: Nestin, B: DCX, C: Ki67. (a: no significant difference, b: difference compared to control group, c: difference compared to STZ group)

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Discussion

The results of the present study showed the positive effect of intranasal administration of insulin in improving cognitive functions in STZ-treated rats showed that these treatments were associated with increased expression of genes involved in neurogenesis in the hippocampus of these animals. In this study, two weeks after intraventricular injection of STZ, rats showed impaired memory acquisition and recall in the EPM test, as well as impaired learning recall in the PA test, while the treatment with intranasal administration of insulin could reduce the mentioned disorders in these animals compared to the STZ group. In confirmation of our findings, many studies have shown that intraventricular injection of STZ causes impairment in learning and different types of memory such as spatial memory, cognitive memory, and passive avoidance memory in rats (2-4, 15, 21). In addition, in line with our findings, previous studies have reported that treating rat models of Alzheimer's disease through intranasal administration of insulin improves memory and learning in these animals (12, 13, 22). Another study has shown that intranasal administration of insulin, without increasing peripheral blood sugar, improves spatial memory in patients with Alzheimer's disease and people with mild cognitive impairment (23). It has also been reported that the intranasal administration of insulin improves spatial memory in rat model of Alzheimer's disease by reducing the expression of amyloid beta (Aβ) protein, reducing neuroinflammation, reducing apoptosis, reducing oxidative stress, increasing the expression of factors involved in the insulin signaling pathway and also increasing the expression of neurotrophic factors (13, 24). In the present study, the results of examining the motor activities of the animals, before and after the injection of the drugs, confirmed that the data obtained from the behavioral tests were not because of the presence of motor disorders in the animals. In line with our results, other studies have also reported the absence of disturbance in animal motor activities following intraventricular injection of STZ and intranasal administration of insulin (3, 18).

The results of this study showed that the intraventricular injection of STZ led to a significant decrease in the expression of genes involved in neurogenesis (Nestin, DCX and Ki67) in the hippocampus of rats, which indicates the effect of STZ in reducing the neurogenesis process. Nevertheless, the treatment of these rats with intranasal administration of insulin showed a significant increase in the expression of genes involved in neurogenesis compared to the STZ group. In agreement with our findings, it has been reported that intraventricular injection of STZ decreases the expression of markers involved in neurogenesis, increases neuroinflammation, disrupts the insulin signaling pathway, and causes cognitive disorders in adult rats (21, 25). In addition, a decrease in neurogenesis has been observed in transgenic mice model of Alzheimer's disease (7, 8). Despite the above studies that have reported a decrease in neurogenesis in animal models of Alzheimer's disease, there are also studies that have shown an increase in neurogenesis in stages of Alzheimer's disease in transgenic mice (6, 26) or in people with Alzheimer's disease (5). However, it has been reported that in cases where the increase of neurogenesis is observed in the brain of patients with Alzheimer's disease, the newly formed neurons do not survive for a long time and are destroyed before maturity (5). The factors involved in increasing neurogenesis in Alzheimer's disease are not precisely known, but it seems that Aβ peptide and amyloid precursor protein (APP) play a role in this process. The neurogenic effects of Aβ peptide have been reported in in vitro and in vivo studies (26, 27) and it has also been shown that APP increases neurogenesis by activating the ERK signaling pathway (28).

Differences in the species, genetics, mutations, stage of the disease, as well as the method of investigating the neurogenesis process can lead to different results in this field, and the increase in neurogenesis in Alzheimer's disease seems to be an ineffective compensatory mechanism to deal with cognitive defects in this disease. Because newly born neurons in the hippocampus, which are involved in hippocampus-dependent learning and memory, will not survive for long (5). Nevertheless, many evidences indicate that neurogenesis defects are one of the factors involved in reducing cognitive functions in Alzheimer's disease

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(7, 8, 21, 25). In the present study, in line with the results of insulin treatment, there are reports of a direct relationship between increased activity of the insulin signaling pathway and neurogenesis. It has been shown in a study that during brain development, the amount of insulin and its receptors in the brain increase significantly (29). Another study has also shown that the incubation of fetal neurons in an insulin-free environment does not change the rate of proliferation, differentiation and survival of neurons, while adding insulin to the culture medium of these neurons, a significant increase in these cases is observed (30). It has also been reported that the amount of neurogenesis in the dentate gyrus decreases in old monkeys, while insulin injection can prevent this decrease (31). Other studies have also shown that intranasal administration of insulin can improve memory and learning in rats with cognitive disorders by increasing mitochondrial biogenesis, increasing the expression of factors involved in the insulin signaling pathway, reducing inflammation, modulating synaptic plasticity, increasing synaptic transmissions and enhancing neurogenesis (18, 32).

The findings of this study showed that the intraventricular injection of STZ caused impairment in memory acquisition and memory recall in the EPM test, as well as impairment in learning recall in the PA test, and these events were associated with reducing the expression of genes involved in neurogenesis in the hippocampus region. However, the intranasal administration of insulin significantly increased learning and memory ability and increased the expression of genes involved in neurogenesis in the hippocampus of STZ-treated rats. Overall, there is the possibility that insulin has improved the cognitive disorders in these animals by modulating the neurogenesis process in the hippocampus of STZ-treated rats. Therefore, insulin can be considered as a promising therapeutic agent in neurodegenerative diseases such as Alzheimer's disease.

Conflict of interest: The authors declare that there is no conflict of interest.

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