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Research Article

Memory Function and Total Pyramidal Neuron Number of Hippocampus in Streptozotocin-induced Diabetic Rats

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Summary

Introduction: Cognitive impairments caused by diabetes mellitus are well documented by previous studies. The studies about neuronal deficits and cognitive impairments in animal models of diabetes mellitus do not simultaneously indicate the total neuron number in the brain subfields and cognitive test performance. In this study, we simultaneously demonstrated total pyramidal neuron number of hippocampus and water maze test performance in streptozotocin-induced diabetic rats.
Material and method: Male Wistarrats were randomly divided into three groups: Control, Sham and Diabetic. Diabetes was induced by intraperitoneal injection of60 mg/kg streptozotocin and blood glucose levels were tested in all rats three and ten days after injection. The rats with blood glucose levels above 300 mg/dl were declared diabetic. Six weeks after induction of diabetes, a version of Morris Water Maze test was applied to evaluate cognitive impairments. All rats were sacrificed for stereological procedures after the test, and total pyramidal neuron number of hippocampus was estimated by using stereological methods.
Results: Streptozotocin induced diabetic rats had a significantly lower total pyramidal neuron number in both CA1 and CA3-2 regions of the hippocampus than that of normal control and sham group rats. Additionally, learning and memory impairments in the Morris Water Maze test accompanied by reduced hippocampal pyramidal neuron number in diabetic rats.
Conclusion: Results of our present study revealed that reduced hippocampal pyramidal neuron number, which is triggered by streptozotocin-induced diabetes, leads to learning and memory impairments in rats.

Key words: Hippocampus, Diabetes,Memory impairment, Stereology, Optical fractionator method, Morris Water Maze

Streptozotosin ile İndüklenmiş Diyabetik Sıçanlarda Hipokampus Total Pyramidal Nöron Sayısı ve Hafıza Fonksiyonu

Özet

Giriş: Diabates mellitus'un neden olduğu kognitif bozukluklar daha once yapılan çalışmalarla gösterilmiştir. Hayvanlarda oluşturulan diabet modellerinde yapılan sinirde fisitleri ve kognitif bozukluklara yönelik çalışmalarda, beyin alanlarının toplam nöron sayısı ve kognitif test performansı birlikte gösterilmemiştir. Bu çalışmada streptozotosin ile uyarılmış diyabet

modeli oluşturulan sıçanlarda, aynı zamanda, su tankı testi performansı ve hipokampusa ait toplam pyramidal sinir hücresi sayısı birlikte gösterilmiştir.
Gereçveyöntem: Erkek, Wistar sıçanlar rastgele üç gruba ayrıldı: Kontrol, yalancı operasyon ve diyabetik grup. Diyabetik grupta bulunan sıçanlarda, intraperitoneal olarak 60 mg/kg streptozotosin enjeksiyonu ile diyabet oluşturuldu ve enjeksiyodan sonraki 3.ve 10. Günlerde sıçanların kan glukoz düzeyleri ölçüldü. Kan glukoz düzeyleri 300 mg/dl üzerinde olan sıçanlar diyabetik Kabul edildiler. Streptozotosin uygulamasından 6 hafta sonar kognitif bozukluğun değerlendirilmesi için modifiye Morris su labirenti testi uygulandı. Testin tamamlanmasından sonar tüm sıçanlar dekapite edildi ve stereolojik yöntemlerle hipokampus toplam pyramidal nöron sayısı hesaplandı.
Bulgular: Streptozotosin ile diyabet oluşturulan sıçanların, normal control ve yalancı operasyon gruplarına gore hipokampus CA1ve CA3-2 bölgelerinde istatistiksel olarak anlamlı düzeyde daha düşük toplam pyramidal nöron sayısına sahip olduğu gösterildi. Buna ek olarak, diyabetik sıçanların hipokampal pyramidal nöron sayısında azalmaya Morris su labirenti testindeki öğrenme ve bellek bozukluklarının eşlik ettiği saptanmıştır.
Sonuç: Bu çalışmamızın sonuçları, sıçanlarda streptozotosin ile oluşturulan diyabetin tetiklediği hipokampal pyramidal nöron sayısındaki azalmanın, öğrenme ve bellek bozukluklarına neden olduğunu ortaya koymaktadır.

Anahtar Kelimeler: Hipokampus, Diyabet, Bellek Bozukluğu, Stereoloji, Optik parçalama yöntemi, Morris su labirenti

INTRODUCTION

Relationship between diabetes mellitus (DM) and cognitive impairment (CI) is well known. Cross-sectional and longitudinal studies on human population provide much evidence in this manner[6,10,29]. Relative risks (RR) of Alzheimer's disease, vascular dementia and any dementia in diabetics are higher than those of nondiabetics(RR: 1.46, 2.48 and 1.51, consecutively) according to a review by Cheng et al., who carried out a meta-analyses of studies on DM and CI[7]. The mechanisms of CI are controversial. Some of the factors claimed to be responsible for CI induced by DM, are hypofunction of insulin-degrading enzyme, mitochondrial dysfunction of hippocampal pyramidal neurons, facilitation of the induction of long-term depression and the inhibition of the induction of long term potentiation in hippocampus, and deficits of hippocampal synaptic plasticity[3,4,11,37]. Additionally, brain magnetic resonance scan conducted in diabetic patients indicates several cerebral changes, including cortical and sub-cortical atrophy, as well as white matter lesions[9]. Cortical atrophy and white matter lesions are quite likely to be a result of induced neuronal death and vascular pathology triggered by DM. In any event, DM possesses the risks of CI, and this fact has been also shown in animal models of DM[18,37].

Animal models of DM contribute to understanding the patho-physiology of the effects of diabetes on the brain. Streptozotocin (STZ)-induced model of Type I DM is one of theanimal models of DM[31]. In this model, peripheral administration of STZ causes a collapse of pancreatic β cells that results in severe hypo-insulinemia and hyperglycemia. Intracerebroventricular or intraperitoneal injections of STZ cause neuronal injury and CI in rats[25,32,35].

Quantitative neuronal deficits is one of the possible reasons for CI caused by DM in diabetic patients or experimental animal models[2,14,25,32,37]. To the best of our knowledge, previous studies about neuronal deficits and CI in animal models of DM have not simultaneously indicated the total neuron number in the brain subfields together with the cognitive test performance[2,14,35]. In our study, we

simultaneously demonstrated total hippocampal pyramidal neuron number and water maze test performance in STZ-induced rat model of DM. The data from the Morris Water Maze (MWM) test and the stereologic investigation showed that STZ-induced diabetes leads to impairments in learning the location of the platform, and decreases total neuron number in the pyramidal layer of the hippocampus.

MATERIAL AND METHODS

Animals

Male Wistar rats weighing 250–300 g (Pamukkale University Experimental Animal Laboratory, Denizli, Turkey) were used in this study. The rats were housed four to five per plastic cage (42×26×15 cm) with sawdust bedding and ad libitum food and water. Animal housing, care and experimental procedures complied with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (NIH Publication No. 85-23) and approved by the Pamukkale University Ethics Committee of Animal Care and Usage.

Experimental design

Animals were weighed and randomly divided into three groups: Control (n=6), Sham (n=6) and Diabetic (n=6).

The diabetic group was treated with 60 mg/kg streptozotocin (Sigma, S-0130) dissolved in 0.5 ml physiological saline (pH=4,5-5,5) intra-peritoneally. Sham groupreceived intraperitoneal injection of the same volume of physiological saline. Control group had no treatment. Tail vein blood glucose levels were tested (Clever Check TDCC 4222 glucometer; strip no: 14) in all rats three and ten days after treatment. The rats with blood glucose levels above 300 mg/dl were declared diabetic. After application of MWM test,all rats were sacrificed for stereological procedures (Figure 1).

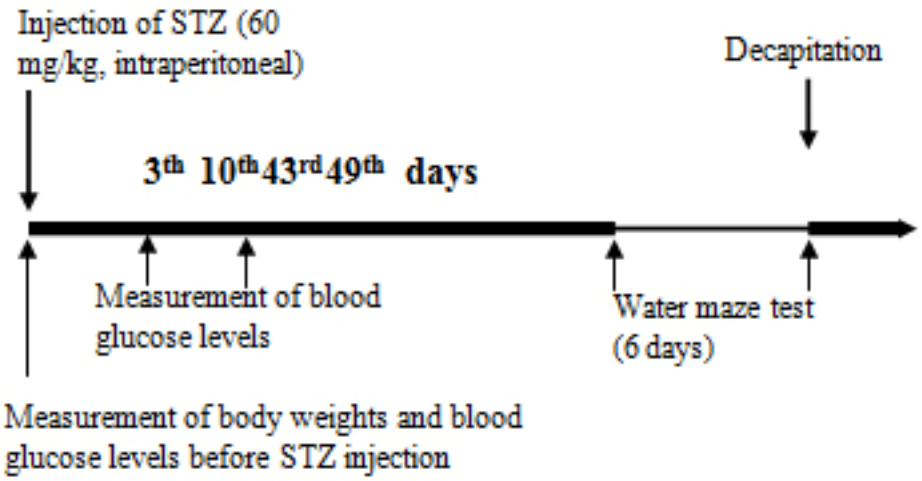


Figure 1: *Schematic view of the experimental design. Stereological procedures were applied after decapitation.*

Morris Water Maze Test

A version of MWM test was applied during the 43-49 days after the treatment, as described previousyly[23,35]. The water tank was filled to a depth of 45 cm with water (22° C). The posters were positioned within sight of the rat in the water tank, and they were maintained in fixed position during the test. The outside surface of the tank was marked four virtual directions as north (N), west (W), east (E) and south (S). Thus the tank was divided into four virtual quadrants, northwest (NW), northeast (NE), southwest (SW), southeast (SE). A clear plastic platform (12 cm × 12 cm) was placed in the center of any quadrant of the water tank, submerged 1.5 cm below the surface of the water, and it was kept in the same position (target quadrant) during the task. The rats were allowed to swim (60 s) to habituate to the water tank without the platform one day prior to training and, were trained for five consecutive days. Each rat was given four trials per day with an inter-trial interval of approximately 10 min for each rat. Every rat was handled before the first trial then it was carefully placed in the water facing the wall of the tank. The order of the release positions was varied systematically throughout the experiment as follows: day 1: N, W, E, S, day 2: W, E, S, N, day 3: E, S, N, W, day 4: S, N, W, E, etc. Each trial began with the placement of the rat in the water facing the tank wall and ended when the rat had escaped onto the platform. If the rat failed to reach within maximal allowed time (60 s), it was gently placed on the platform and allowed to remain there for 15 s.

The experiments were recorded via camera placed on the ceiling. The output of the camera was captured by the tracker (Noldus, Ethovision, NL), and analyzed by using the Ethovision 3.1 software.

Performance of the animal in each trial was assessed by three parameters; swimming time to reach the platform (Escape Latency; EL), swimming distance to reach the platform (Path Length PL) and swimming speed (Velocity; V).

Probe Trial

The hidden platform was removed from the pool and a probe trial was performed to assess the degree of memory consolidation that had taken place after learning on the sixth day. Each animal was released from N starting position and trial ended after 60 s. The elapsed time in the target quadrant was recorded and analyzed.

Stereological Cell Counting

The animals were sacrificedafter MWM test, and brains were removed by craniotomy and immediately frozen in cryostat chamber (Leica CM3050,Bensheim, Germany). The frozen brains were cut in horizontal planes at a thickness of 150 μm. The serial brain sections collected according to systematic random sampling rules were stained with Hematoxylin–Eosin[36]. The first section in the series to be analyzed was chosen at random from the first three sections, and thereafter every third section was chosen for analysis. Thus “section sampling fraction” (ssf) was 1/3.

Real time microscopic images were transferred to a monitor by using a microscope-camera-monitor complex (Olympus research microscope CX31, 100× oil objective, Numerical Aperture=1.25, USA; Sony LCD monitor LMD-2010, Tokyo; Sony Color Video Camera SSc-DC88P, Tokyo). The optical fractionator was used to count and estimate total number of pyramidal neurons in the hippocampus. Optical fractionator method, described West et al., is a combination of optical disector and fractionator methods[36]. Ten or more brain sections including the hippocampus were used for pyramidal neuron counting.

The thicknesses of every brain section and disectors were measured by using a microcator (Heidenhain MT12, Germany)

mounted on the microscope stage, and mean section thickness was calculated for each brain.

Stepping on x–y axes on microscopic images of the pyramidal layer of hippocampus was performed according to the method described by Adiguzel et al[(1)]. A transparent grid in size 50X50 μm^2 was placed on the screen for stepping. Ten grid areas (25000 μm^2) were passed from a random starting area for cell counting on the hippocampus image which was transferred to the screen. Then, an unbiased counting frame (20X20 μm2), described by West et al[(36)], was super-imposed on the screen. Thus the proportion of the unbiased counting frame area to the area associated x, y step, "area sampling fraction" (asf) was 400/25000 μm^2 (4/250). To avoid overestimation, the counting frame was focused through 30 μm (optical disector height) started after 5 μm from the bottom surface of the tissue. Also the neuronal nuclei hit into the counting frame were counted according to unbiased counting rules. The proportion of the height of the optical disector to the mean thickness of the section was calculated as "thickness sampling fraction" (tsf = optical disector height (30 μm) /mean section thickness).

Total pyramidal neuron number (TPNN) was estimated by the formula described below:

$TPNN = Q^- \times [1/ssf] \times [1/asf] \times [1/tsf]$

Q^-: the total number of neurons counted in the disectors on the sampled sections;

ssf: the section sampling fraction;

asf: the area sampling fraction;

tsf: the section thickness sampling fraction;

Coefficient of error (CE) was also estimated as described in the literature[(12)].

Statistical Analysis

Data were presented as mean ± standard error of the mean (S.E.M.). Between group differences in body weights, blood glucose levels, stereological cell count and water maze test (EL, PL and V) data were analyzed by a one-way analysis of variance (ANOVA) with Post Hoc Bonferonni test. A p-value of less than 0.05 was considered to be statistically significant in all statistical analyses.

RESULTS

Body Weights and Glucose Level:

Diabetic group showed symptoms of diabetes including polyuria and polydipsia (based on frequency of cage cleaning and amount of water bottles consumed per day).There were no statistically significantdifferences among the mean of blood glucose levels of all groups at the beginning of the experiment(Control: 118±5.3 mg/dl; Sham: 119±4.9 mg/dl; Diabetic: 116±3.8 mg/dl, p>0.05). Blood glucose levels were above 300 mg/dl in all diabetic rats at the 3rdand the 10th days after STZ administration (the 3rd and the 10th days respectively, Control: 115.3±6.2/118.5±6 mg/dl; Sham: 117±4.9/117.9±3.4 mg/dl; Diabetic: 443±9.6/457.7±7.1 mg/dl; p=0.00 for all comparisons of diabetic group to control and sham groups). The diabetic rats failed to gain body weight, at the end of the experiment their mean body weight was significantly lower than those of control and sham groups (Control: 287.6±11.2 g; Sham: 289.2±8.3 g; Diabetic: 204.5±5.8 g; p=0.00).

Data fromMorris Water Maze Test:

The means of EL and PL decreased gradually during the five days of training in all groups (Figure 2 and 3, respectively) The means of EL and PL were significantly different among the groups[F(2)=4,972 and 6,469; p=0.007 and 0.002, respectively]

Although the mean EL decreased gradually in all groups, post-hoc analyses revealed that diabetic group had a highermean EL than that of the control and sham groups (p=0.03 and p=0.01, respectively). Although the mean PL decreased gradually

in all groups, post-hoc analyses revealed that diabetic group had a higher mean PL than that of the control and sham groups (p=0.01 and p=0.00, respectively).Diabetic group tended to spent more time and swam a greater distance to reach the platform.

There was no statistically significant differencein terms of V amongthe groups (p>0.05) (Figure 4). This finding suggests that, the motorfunctions (ability to swim)did not affectthe MWM test.

Data from one-day probe trial,performed to evaluate how well the animals consolidate the location of the platform during the test, indicated no statistically significant difference between the groups (p>0.05; Figure 5).

The total pyramidal neuron number of hippocampal CA1 and CA3-2 regions

The total neuron numbers in both CA1 and CA3-2 regions of the hippocampus were significantly different among the groups [F(2): 32,663 and 65,698, respectively; p=0.000 for both]

Post-hoc analyses revealed that the total pyramidal neuron number in both CA1 and CA3-2 regions were significantly lower in diabetic group (70641 ± 8616; 129481 ± 13326, respectively) than those of the control (121301 ± 11201; 237663 ± 18856) and sham (115778 ± 15031; 210351 ± 18274) groups (p=0.00 for all comparisons of diabetic group to control and sham groups; Figure 6, Figure 7, Figure 8). Coefficient of error (CE) values werein acceptable rangein all subjects[12].

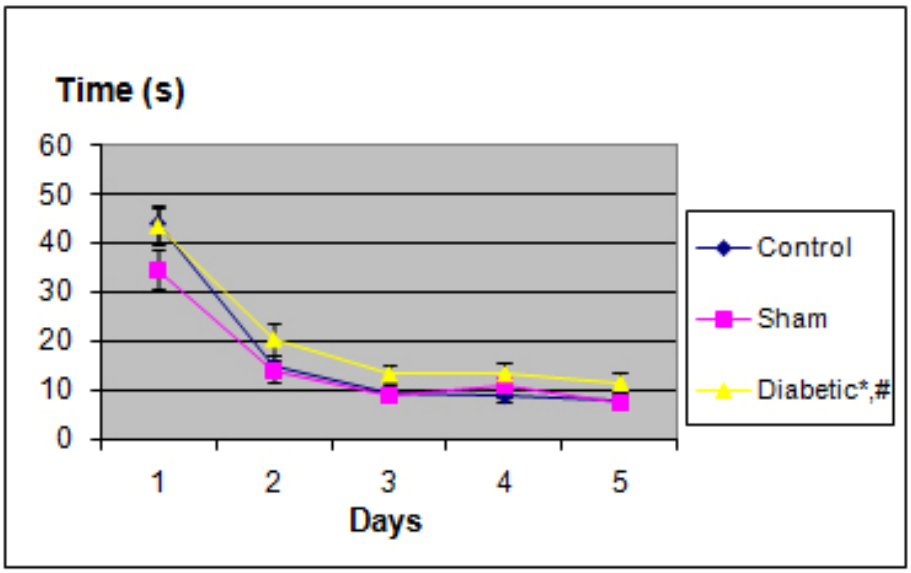


Figure 2: *The line graphic shows the means of EL in MWM test. In all groups, EL decreased gradually during five days of training. The rats in all groups had learned how to find platform. However, during the MWM test, diabetic rats spent more time to find the platform (vs. control *p=0.03, vs. sham #p=0.01).*

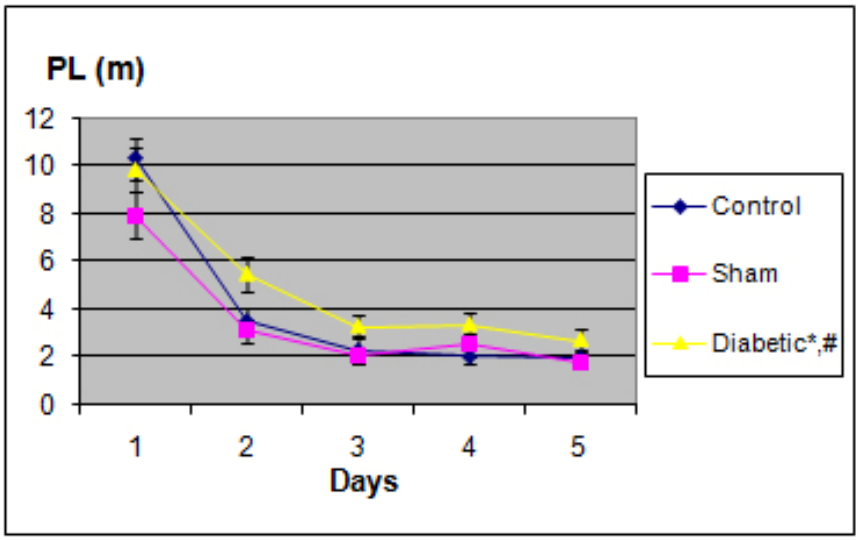


Figure 3: *The line graphic shows the means of PL in MWM test. In all groups PL decreased gradually during five days of training. The rats in all groups had learned how to find platform. However, during the MWM test, diabetic rats swam more distance to find the platform (vs. control *p=0.01, vs. sham #p=0.00).*

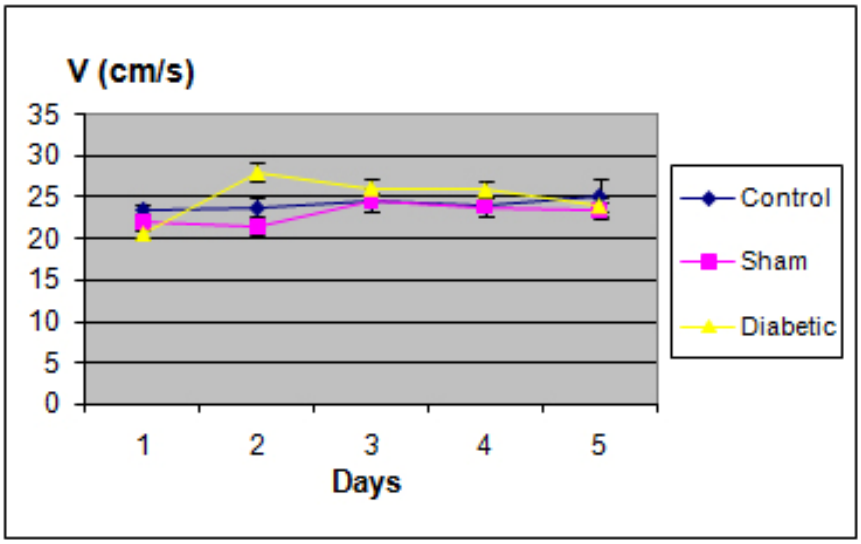


Figure 4: *The line graphic shows the means of V in MWM test. There were no statistically significant differences among groups (p>0.05).*

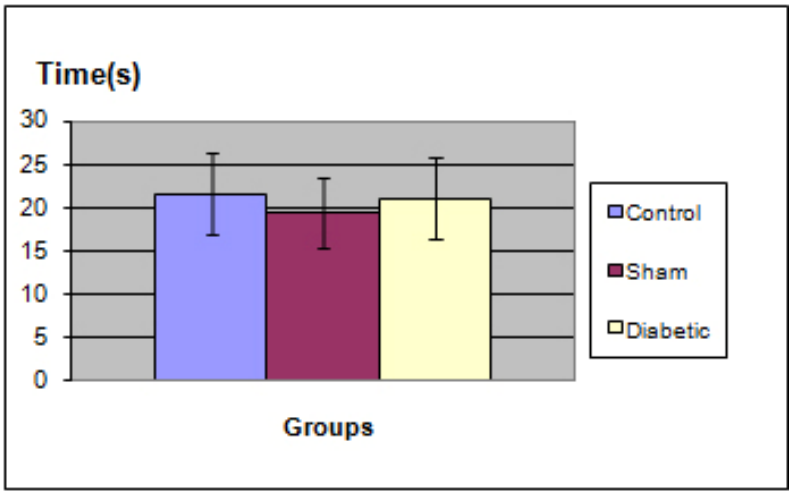


Figure 5: *The bar graphic of data (time elapsed in the target quadrant) from the one-day probe trial of the Morris Water Maze Test. There were no statistically significant differences among groups.*

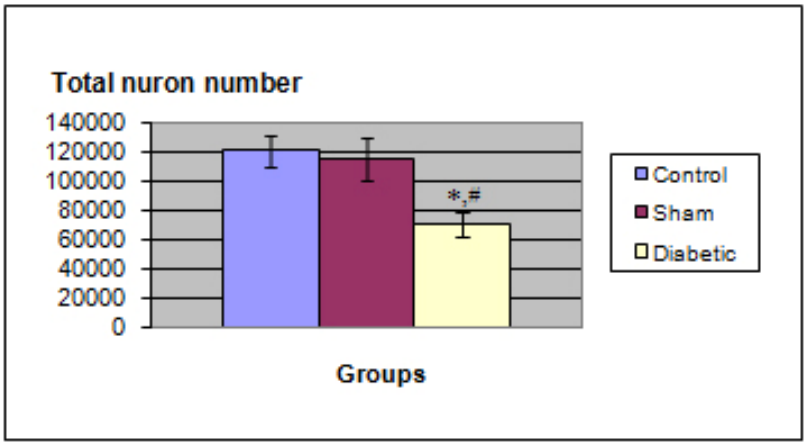


Figure 6: *The total neuron number of hippocampal CA1 region. Neuron number was significantly decreased in diabetic group (vs. control *p=0.00, vs. sham #p=0.00).*

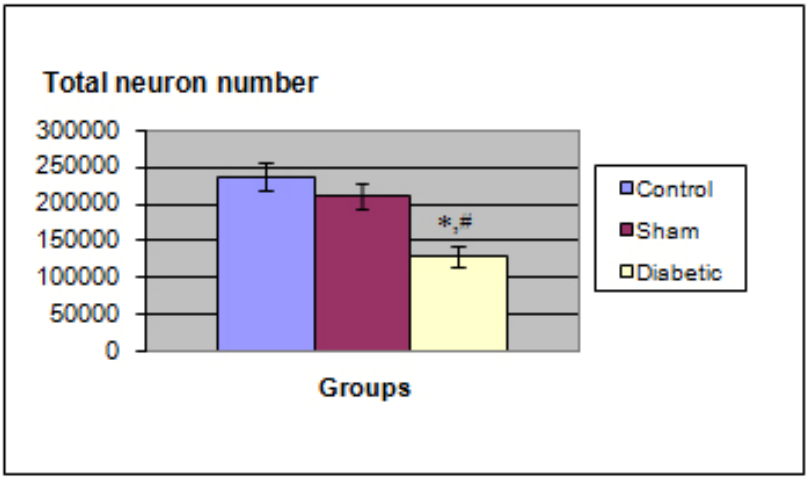


Figure 7: *The total neuron number of hippocampal CA3-2 region. Neuron number was significantly decreased in diabetic group (vs. control *p=0.00, vs. sham #p=0.00).*

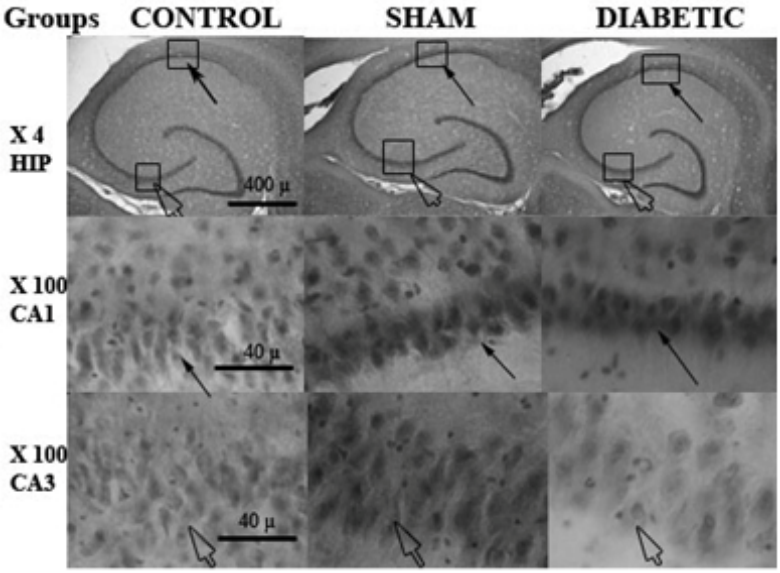


Figure 8: *Comparative pictures of the hippocampal horizontal sections stained by Hematoxylin–Eosin. The columns included in the pictures belong to control, sham and diabetic groups. The pictures in the first row are hippocampus sections in 4x magnification, closed arrows indicate CA1 region represented in the second row, open arrows indicate CA3 region represented in the third row in 100x magnification. There are no qualitative differences among the groups.*

DISCUSSION

In this study, changes in both total hippocampal pyramidal neuron number and learning-memory performance were studied in STZ-induced diabetic rats. Our results showed that STZ-induced diabetes impairs MWM test performance and reduces total hippocampal pyramidal neuron number in rats. Hyperglycemia due to diabetes mellitus is an important endogenous oxidative stress factor, harmful for sensitive tissues such as brain. The relationship between diabetes mellitus and cognitive impairments is well known[5].On the other hand, the mechanisms that underlie the pathophysiology are controversial,and so the basic experimental animal researches on diabetes mellitus have importance nowadays. STZ-induced diabetes, an animal model of experimental diabetes was preferred in the present study to provide an example of hyperglycemia[20].We used MWM test to evaluate spatial memory[23] and stereological methods[13,36]to evaluate changes inhippocampal total pyramidal neuron number in rats. The results were compared among the control, sham and diabetic rat groups.

Experimentally STZ-induced diabetes models have been used by scientists to study mechanisms, complications and pathophysiology of diabetes since 1960s due to their resemblance to diabetes in humans[16]. Single dose STZ, N-nitroso derivative of glucosamine, has a massive pancreatic beta cell destruction effect in rodents. Diabetic activity of STZ has a dose related effect, suchthat lover doses (between 40 and 55 mg/kg body weight) cause diabetes like totype 2, and higher doses (>60 mg/kg body weight) cause diabetes like to type 1 in humans[15,16]. Chemically induced diabetes animal models have toxic effects on kidney and liver, but STZ has less side effects compared to the other chemicals such as alloxan[15,16]. Additionally, genetically diabetic rats are expensive in price and not easily available for researchers.On the

other hand it is impossible to gain insulin resistance via STZ-induced diabetes model, so this model is not appropriate to investigate type 2 diabetes.According to literature, our results should be considered as an acute effect of the type 1 diabetic stress.

In the present study, the STZ-induced diabetic rats had higherblood glucose levels and lower body weights when compared to control and sham group rats. This finding is necessary for success of the experimentally induced diabetes model. Otherwise these animals are not diabetic under normal condition[15]. Although, polyuria and polydipsia are other symptoms of diabetes these findings are not primary predictors of DM. Therefore water and food consumptions of rats wasnot strictly measured in our study.

MWM test allows testing spatial learning, the learning abilities of the animals including locational parameters such as distances and directions[21,23]. Hippocampal lesions impair learning and memory either in experimental animal models or in humans[30,33]. Additionally, the hippocampus has an important role in navigation[24]. Morris et al showed that hippocampally damaged rats have impairments in spatial learning tested by MWM[22]. On the other hand,De Hoz et al indicated that large lesions of the hippocampus with small remnants of hippocampal tissue impaired not completely but partially the rate of spatial learning and one-trial spatial memory in rats[8]. According to De Hoz et al, more hippocampal tissue is required for successful performance in MWM test[8]. It seems that MWM test used in the present study was useful to evaluate the spatial learning in rats with chemically induced diabetes.But, the absence of delayed matching-to-place task in MWM test may be considered as weakness of our study.

Although, optical fractionator method, a stereological method, allowed us to estimate total pyramidal neuron number of hippocampus which is important for rapid and successful performance in MWM test in rats[23],the number of pyramidal neuronsalone is not enough to explain the pathophysiological basis of the impairment of learning and memory in chemically induced diabetic rats. It is known that changes in cellular ultrastructure, potentiation mechanisms and synaptic plasticityaccompany neuronal lose in diabetic rats[3,17,37]. However, proper methods are needed to evaluate these types of changes.Difficulties in application of multi methods in the same animal group prevented us from applying the other methods.

MWM test uses the natural swimming behavior of the animal[23,28].Intact motor function of the animals is necessary to evaluate impairment of spatial memory. This parameter is measured by velocity in MWM test[23]. In our study, there were no statistically significant differences among the groups in terms of the velocity. According to this result, it can be stated that motor performance of the rats did not affect the MWM results in our study.

The data obtained from MWM test about spatial learning and memory showed that STZ-induced diabetic rats had slower learning performance than that of the sham and the control groups in our study.Consistent with our results, previous studies indicated impaired learning performance in STZ-induced diabetic rats.[3,4,17,19,27,37].In these aforementioned studies, different tests such as t-maze, MWM, can test, y-maze etc. were used to evaluate learning and memoryThe results of these tests consolidated the effect of STZ-induced diabetes on learning and memory in rats.

The reduction of total pyramidal neuronal numbers in the hippocampus in diabetic rats is another result of our study. This result shows that diabetes has effects on neuronal survival or neurogenesis or both. Zhao et al. reported that diabetes increased the expressions of Bax and caspase-3,

which led to the apoptosis of the CA1 pyramidal neurons[38]. Another study indicated translocation of Bax from cytoplasm to mitochondria and cytochrome c release into the cytoplasm from mitochondria triggered by STZ-induced diabetes in rats[37]. So, the hippocampal neuronal reduction in diabetesmay be explained by apoptosis.

We found neuronal reduction in bothCA1 and CA3-2 regions of hippocampus. Hippocampal tissue loss may play an important role on learning and memory function of the hippocampus[8]. On the other hand, recent studies indicated that cortical and subcortical structures other than the hippocampus alsohave an important role in learning and memory[26,34]. In diabetic rats, learning and memory impairment may not onlybe related to the reduced number of pyramidal neurons in hippocampus but also the impaired function or tissue loss of the cortical and subcortical structures. Further investigations are needed to understand the mechanisms of learning and memoryfunctionsrelated to the hippocampus and other cortical and subcortical structures.

In summary, the present study revealed that STZ-induced diabetic rats had statistically significant lower total pyramidal neuron number in both of the CA1 and CA3-2 regions of the hippocampus compared to normal control and sham groups. Additionally learning and memory impairments shown by MWM test was accompanied by reducednumber of hippocampal pyramidal neurons in diabetic rats. These results may be interpreted as the reducing of hippocampal pyramidal neuron number, which is triggered by STZ-induced diabetes, leads to impairments of learning and memory function.

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