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Original Article

Can melatonin correct the negative effect of experimental diabetes created during the maternal period on fetal rat development and puppies cognitive functions?

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Abstract

Aim: Chronic hyperglycemia can cause cognitive impairments such as learning and memory impairment. In our study, we aimed to investigate the levels of glial fibrillary acidic protein (GFAP), neural cell adhesion molecules (NCAM), lipid peroxidation (LPO), and glutathione (GSH) molecules and the protective effect of melatonin in the brain tissue of baby rats with diabetic mothers.

Materials and Methods: Wistar-Albino rats used in the experiments were obtained from Firat University Experimental Research Center. Morris Water Maze Test is a learning and memory test commonly used in rats and mice. In the statistical analysis of the data; one-way analysis of variance (One-way ANOVA) was used to evaluate the significance of NCAM, GFAP, LPO, GSH levels between three groups, and repeated measures analysis of variance (Repeated measures one-way ANOVA) was used to evaluate the Morris Water Maze learning test.

Results: Learning was worse in rats whose mothers were diabetic compared to diabetes + melatonin and control groups. With the administration of melatonin to diabetic mothers during their pregnancy, an improvement was observed in the learning ability of baby rats. NCAM 180, GFAP, GSH levels were significantly lower ($p < 0.05$, $p < 0.001$, $p < 0.05$), and LPO level was higher ($p < 0.001$) in baby rats with diabetic mothers compared to the control group. NCAM 180 and GFAP levels were significantly higher in the group that was administered melatonin during pregnancy ($p < 0.05$, $p < 0.01$), and LPO levels were lower ($p < 0.01$). With the administration of melatonin during pregnancy, GSH levels were higher than the diabetes group, even though the difference was not statistically significant.

Conclusion: Learning and memory functions are impaired in the offspring of diabetic mothers. The decrease in NCAM isoforms can inhibit brain development and the formation of synaptic plasticity. Decreased GFAP density may pose a problem in completing brain maturation in offspring of diabetic mothers. It has been observed that the administration of melatonin to diabetic mothers during their pregnancy is protective against the harmful effects of oxidative stress in their offspring due to its antioxidant effect.

Keywords: Diabetes mellitus; GFAP; learning; melatonin; NCAM

INTRODUCTION

Diabetes is a chronic metabolic disorder in which the organism cannot make enough use of carbohydrates (HR), fat, and proteins due to insulin deficiency or insulin effect and requires continuous medical care. Depending on the pathogenesis, diabetes can be classified as type 1, type 2, gestational diabetes mellitus (GDM), or other types of diabetes. GDM is usually diagnosed during the second or third trimester of pregnancy, and its prevalence varies between 1% and 17%, depending on the population studied and the diagnostic test used (1). The impact of diabetes on maternal and infant health, including the developmental nature of the disease, has been studied in detail. According to the Hyperglycemia and Adverse Pregnancy Outcomes study, associations between changing gestational glycaemia and adverse health problems in offspring exist even during the prediabetes, raising critical health concerns such as glucose management during pregnancy. Potential pathways linking maternal diabetes with health outcomes such as adiposity, cardiometabolic health, and cognitive performance have been widely reported in numerous studies (2). The fetal environment in maternal diabetes is mainly characterized by hyperglycemia, chronic hypoxia, and iron deficiency. Moreover, altered

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glycaemia during pregnancy can have a major impact on fetal neurodegenerative disorder and offspring's cognition as well as increase the risk of having mental disorders such as Attention Deficit and Hyperactivity Disorder. However, epidemiology cohort studies have reported very different aspects and hence impaired, unaffected, and even improved cognitive functions in children were exposed to diabetes. To date, no systematic review or meta-analysis is evaluating the relationship between maternal diabetes and cognitive ability in offspring. Therefore, we hypothesize that a diabetic pregnancy may create an intrauterine environment that causes neurodevelopmental impairment of the fetus, thus causing critical limitations in future cognitive abilities in infancy or childhood (2). In previous human and animal studies, DM was found to be associated with pathological changes in the central nervous system, causing a decrease in cognitive function, behavioral disorders, and an increased risk of vascular abnormalities in the brain. Many structural brain changes such as increased hippocampal astrocyte reactivity, abnormal synaptic plasticity, vascular changes, decreased dendritic complexity, and impaired neurotransmission have been described (3). In the pathogenesis of diabetes-induced brain damage, impaired double electron transport systems are caused by damaged mitochondria, which are the main focus of reactive oxygen systems (ROS) in neurons. Therefore, it is considered to play a fundamental role in the pathogenesis of diabetic complications due to oxidative stress, increased free radical production, and abnormal antioxidant defenses. Melatonin (N-acetyl-5-methoxytryptamine) is the main product of the pineal gland and is also produced in various organs in vertebrates. Melatonin concentrations in serum exhibit a pronounced circadian rhythm with the highest levels during the night and lowest concentrations during the day. In the last five years, intensive research has shown that melatonin plays a role in various modulations of endocrine, neural, and immune processes. Numerous publications have proven that melatonin protects the brain from many chemical attacks both in vivo and in vitro (4).

In our study, we aimed to show that learning and memory functions are impaired in the offspring of diabetic mothers, and melatonin administration to diabetic mothers during pregnancy can be protective against the harmful effects of oxidative stress in their offspring due to its antioxidant effect.

MATERIALS and METHODS

Experimental animals

Wistar-Albino rats used in the experiments were obtained from Firat University Experimental Research Center. The rats were fed in cages that were specially prepared and cleaned every day in an environment with a ventilation system. Feed was given in special steel containers and water in stainless steel ball bottles as normal tap water. Experimental animals were fed with rat feeds in the form of pellets specially prepared in Elazig Feed Factory. The additives in the composition of the feed given to rats are shown in Table 1. The care of the rats continued in this way until the experimental application stage.

Table 1. Composition of feed given to experimental animals

Feed Composition	%
Wheat	10
Corn	21
Barley	14
Bran	8
Soy sauce	25
Fish flour	8
E-bone meal	4
Molasses	4
Salt	4
*Vitamin Mix	1
**Mineral Mix	1

*** Vitamin mix: There are vitamins A, D3, E, K, B1, B2, B6, B12 and nicotinamide, folic acid, D-biotin, and choline chloride in the vitamin mix of feeds given to experimental animals.**
**** Mineral mix: Consists of manganese, iron, zinc, copper, iodine, cobalt, selenium, and calcium**

Experimental Applications

Before starting the experimental studies, a preliminary study was carried out in order to minimize the possible problems. The ambient temperature of the experimental animals was kept constant between 22 and 25 C and the animals were followed under 12 hours of light and 12 hours of darkness. 12 female rats were selected for the study and the mother rats were divided into three groups in equal numbers (n = 3). Four rats each were taken as the first group diabetes (induced by STZ), the second group diabetes + melatonin group, and the third group as the control group. The mothers-to-be rats were followed with vaginal smear for fifteen days and their ovulatory cycles were determined. The rats without any cycle disorder were determined and mated. Assuming that rats with sperm detected in vaginal smear will be pregnant, 8 female rats with diabetes were injected intraperitoneally with 40 mg/kg STZ. Blood glucose levels were measured from the tail vein of rats 48 hours after STZ injection using a diagnostic glucose kit. Rats with blood glucose of 200 mg / dL and above were considered diabetic. In order to avoid problems with high glucocorticoid levels, which are known to have long-term effects on the behavior and movements of the offspring, glucose monitoring was not performed on STZ-treated mother rats during gestation. Diabetic rats were administered subcutaneously a single morning dose of 5 U / kg/day NPH insulin in the last three days of pregnancy, as used by Kinney et al. (5).

Two pregnancies initially occurred in the diabetes group, one pregnancy in diabetes + melatonin group, and four pregnancies in the control group. New pregnancies were made in diabetes and diabetes + melatonin groups. Pregnancy was established in four rats in each group. Their pregnancies were normal. A total of 32 infant rats were born in the diabetes group, 34 in diabetes + melatonin group, and 36 in the control group. In their

follow-up, 6 of the baby rats in the diabetes group, 5 of diabetes + melatonin group, and 2 of the control group died. Immediately after birth, total brain dissection was performed for seven offspring from diabetes, melatonin, and control group, and dissected total brains were frozen in dry ice to study GFAP, NCAM, LPO, and GSH. It was stored at –80 C until the analysis was done. The baby rats were fed by their mothers in the same cage until they were 1 month old; no treatment was given to the mother for diabetes during that period. At the end of the first month, the offspring were separated from their mothers and fed in different cages according to their gender. For the Morris Water Maze test, 7 adult male rats (2 months old) whose mothers were diabetes (STZ group), 7 adult male rats (2 months old) whose mothers were diabetes and melatonin was administered (STZ + melatonin group), and 7 adult male rats (2 months old) whose mothers were normal (control group) were taken. After 75 days, Morris Water Maze learning test was applied to all groups.

Morris Water Maze Test

Morris Water Maze Test is a learning and memory test commonly used in rats and mice (6). Morris's water tank is a circular galvanized tank, 120 cm in diameter and 50 cm high. The water tank was filled with 25 cm of water and the water was dyed to black to prevent a black platform of 10x10 cm, which was left 2 cm below the water from being seen. The temperature of the water was kept constant at 24±2 C degrees. The location of the tank and the platform location were kept constant throughout the experiment. A visual cue was placed outside the tank so that the subjects could perceive the spatial position of their location. The water tank was virtually divided into 4 parts and the platform was placed in the middle of one of these quadrants. The rat was dropped into one of the other 1/4 areas and allowed to swim and find the platform within 60 seconds. When he found the platform, he was allowed to rest there for 30 seconds, and then he was taken and kept in a separate cage for 30 seconds. It was left in the water tank again, the same process was repeated 4 times for each animal, and the time to find the platform was recorded. The rat that could not find the platform within 60 seconds was taken and left on the platform and allowed to rest for 30 seconds. The same trials were made for 5 days for each animal and the times were recorded. To test the memory enhancement process, a probe test was performed 24 hours after the 5-day test. In this test, the platform was removed from the tank, and subjects were floated. Subjects are naturally expected to search more in the quarter of the tank where the platform was previously located. This time measures memory consolidation. The time that the subjects swam in the quarter where the old platform was located was recorded.

Collection of Newborn Rat Total Brain Samples, Analysis by SDS-PAGE and Western Blot

After the experimental applications, the total brains of the newborn rats that were decapitated in accordance with the decisions of the ethics committee were taken; their volumes were determined, and immediately frozen in dry ice. Samples were transferred to tared Eppendorf tubes. Their weights were determined by weighing them on a precision scale and stored at –80 C until analysis. Free and non-free protein samples were analyzed with SDS-PAGE prepared as specified by Laemmli (7). A 10 ml separating gel solution was prepared to be placed between two glasses held in a suitable position to form a gel. This gel solution was thoroughly mixed and transferred between two glass plates, which were squeezed from certain parts by means of a suitable automatic pipette. While the gel was added between the glass plates, a gap equal to the height of the comb teeth (1cm) was left at the top. The gel between these two glass plates in the form of a cassette was kept at room temperature for approximately 30 minutes, and the acrylamide monomers between them were polymerized. Then a comb with the appropriate number of teeth for the sample number was placed on the upper part of the two glass plates. Stacking gel expressed as the intermediate filling material of comb teeth was prepared in 10 ml. The prepared gel solution was thoroughly mixed and the spaces between the combs teeth placed in the gel cassette were filled with the help of a suitable automatic pipette. This filling was completed to the top of the two glasses. Since the stacking gel polymerizes very quickly, attention was paid to the processing in a short time. Polymerization was achieved by waiting at room temperature for 25-30 minutes. The comb was removed from the polymerized gel. During this process, care was taken not to damage the slots in the gel where the samples would be left. The cassette made of glass plates was placed in the electrophoresis tank. Protein solvent solution; It was prepared as 0.125 M Tris (pH 6.8), 2% SDS, 0.002% Bromophenol blue, 20% glycerol, 10% mercaptoethanol. Approximately 150 l of the additional solvent solution was added to each protein sample taken and mixed well. Depending on the width of the comb tooth, 10-20 l of the mixture we prepared was transferred. A sufficient amount of tank solution was added to the tank.

Current with a low voltage (150 V) was applied to electrophoresis before the power supply. The electrophoresis device was turned off when the blue dye band, which was visible to the naked eye, reached the bottom of the gel. After the electrophoresis process was completed, the two glasses forming the cassette were separated from each other and the gel in between was removed. This gel was taken into 1.25% Coomassie blue dye environment in order to make the protein bands visible. It was kept here at room temperature for at least half an hour, at most overnight. The gel taken from the dye solution was taken into the dye removal solution (destaining solution). The dyestuff outside the protein bands was removed with occasional shaking. It was kept in the decolorizing solution for 5 minutes and the solution was poured. The gel was taken back into the decolorizing medium and this process was repeated 2-3 times. Thus,

the dye outside the protein bands on the gel was removed. The photos of the protein bands visible on the gel were taken with the help of a camera. Western blot analysis of newborn rat total brain samples was performed according to the method applied by Baydaş et al. (8). Transfer of proteins in the gel to nitrocellulose membrane (blotting): After the SDS-PAGE was completed; the polyacrylamide gel was taken to be blotted. To achieve the transfer to the nitrocellulose membrane, the polyacrylamide gel and nitrocellulose membrane (Schleicher and Schuell, Inc., USA) were placed face to face with no gaps between their surfaces, and these were placed in a blotting device wrapped with filter papers and saturated with buffer solution. An electric current of 150 mA was applied for 60 minutes for the contrivance placed in the tank filled with the cooled buffer solution. In this way, the transfer of proteins was achieved. Coating non-protein bound regions with unrelated proteins (blocking) on the nitrocellulose membrane to prevent non-specific reactions: Nitrocellulose membranes taken into petri dishes after the blotting process is finished with buffer solution (NaH2PO4.2H2O (0.025 M), Na2HPO4.12H2O (0.075 M), NaCl (1.45 M)] was washed 3 times for 5 minutes on the shaker. Nonspecific binding was blocked with 1% fresh bovine serum albumin in 100 mM NaCl, 20 mM Na2HPO4, 20 mM NaH2PO4 (pH 7.2) buffer at 37 ° C for 90 minutes. Reaction with specific antibodies: Polyclonal rabbit anti-rat NCAM and GFAP antibodies were used as primary antibodies. NCAM and GFAP primary antibody was prepared at a ratio of 1:2000 in buffer containing 0.05% Tween-20 and used. Nitrocellulose membranes were incubated with NCAM and GFAP antibody at +4 C overnight. In the next stage, the nitrocellulose membranes were washed 5 times for 5 minutes with a buffer solution. After the washing process was completed, the nitrocellulose membranes were incubated with goat-anti-rabbit immunoglobulin prepared at a ratio of 1: 1000 in buffer containing 0.05% Tween-20 at 37 ° C for 90 minutes. In the next step, nitrocellulose membranes were washed 5 times with a buffer solution for 5 minutes. Visualization of the bands: A solution of 0.03-0.05% diaminobenzidine prepared in 1 M Tris (pH: 7.4) buffer was used to visualize the bands. As a result of the reaction with diaminobenzidine, the bands on nitrocellulose membranes became visible after a short time. At the end of a 5-10 minute reaction period, the nitrocellulose membranes were thoroughly washed after the bands colored with diaminobenzidine were clearly visible. After the nitrocellulose membranes were dried thoroughly, the relative densities of the bands were taken for analysis. The relative densities of the bands were analyzed using the Lab. Works 4.0 (Ultra-Violet Products Ltd. Combridy, CD4 1TG UK) software.

Lipid Peroxidation, Glutathione Measurement Method in Newborn Rat Total Brain Samples

Tissue LPO; (Malondialdehyde 4-Hydroxyalkenal (MDA + 4-HDA)) levels were determined using the commercial kit LPO-586 (Oxis International, Inc., Portland, USA). GSH levels in the samples were determined using the GSH-400 (Oxis International, Inc., Portland, USA) commercial kit.

Statistical Analysis

Primary outcome measures of this study were calculated with help of experimental power analysis; effect size for total brain weight was 2.13, the amount of Type I error (alpha) was 0.05, and the sample size in the groups was 26, the obtained power (1-beta) was 1 (9). Quantitative data were summarized with mean and standard deviation values. The data obtained were loaded into IBM SPSS Statistics for Windows 26.0 package program and analyzed. The compliance of quantitative data to normal distribution was evaluated by using the Shapiro-Wilk test. In the statistical analysis of the data; one-way analysis of variance (One-way ANOVA) was used to evaluate the significance of NCAM, GFAP, LPO, GSH levels between three groups, and repeated measures analysis of variance (Repeated measures one-way ANOVA) was used to evaluate the Morris Water Maze learning test. After significant one-way analysis of variance, the Tukey test was used in paired comparisons and the Bonferroni test was used after analysis of variance in repeated measures. $P < 0.05$ values were considered significant. Data were expressed by appropriate graphs according to days or groups.

RESULTS

At the beginning of the study, four female rats were selected in each group. Initially, two pregnancies occurred in the diabetes group, one pregnancy in diabetes + melatonin group, and four pregnancies in the control group. New pregnancies were made in diabetes and diabetes + melatonin groups. The pregnancies of the rats were normal. The blood glucose level was 231 ± 3.2 mg / dL in the diabetes group, 210 ± 3.4 mg / dL in the diabetes + melatonin group, and 120 ± 3.1 mg / dL in the control group. Their pregnancies were normal. A total of 32 infant rats were born in the diabetes group, 34 in diabetes + melatonin group, and 36 in the control group. In their follow-up, 6 of the baby rats in the diabetes group, 5 of diabetes + melatonin group, and 2 of the control group died.

GFAP, NCAM, LPO, and GSH were studied by decapitating seven babies born in diabetes, diabetes + melatonin, and control groups.

The Morris Water Maze Test included 7 male adult rats whose mother had experimental diabetes, 7 adult rats whose mother was diabetic and treated with melatonin, and 7 adult rats whose mother was normal.

The body and total brain weights of the experimental groups are shown in Table 2.

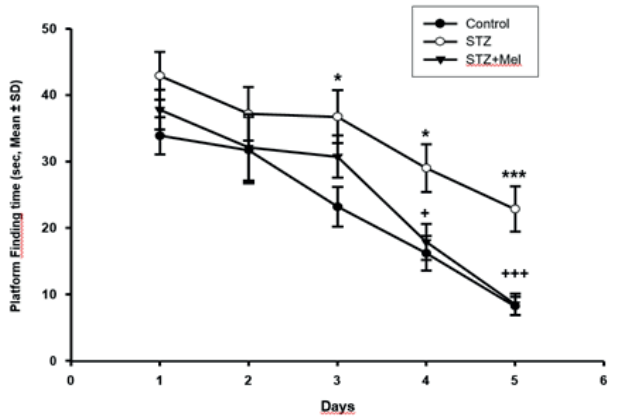
Table 2. Average body and total brain weights of the experimental groups

Variable	Control	Diabetes	Diabetes + Melatonin
Body weight (gr)	171 ± 9.3	211.8 ± 6.3	200 ± 5.2
Total brain weight (gr)	1.76 ± 0.07	1.92 ± 0.08	1.81 ± 0.07

Morris Water Maze Learning Test Results

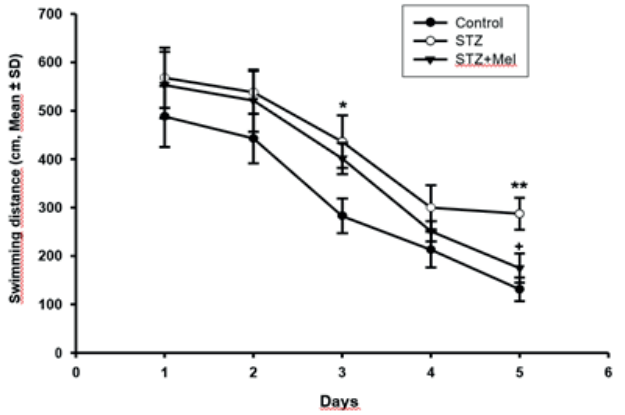
It was observed that learning ability gradually increased from day 1 to day 5 in diabetes, diabetes + melatonin, and also in the control group. The times to find the platform of diabetic mother-offspring (STZ group rats) were significantly longer on the 3rd, 4th, and 5th days compared to the control (the longer the time means that the learning was worse; p <0.05, p <0.001). Melatonin administration during pregnancy significantly corrected the deterioration in behavioral performance in the STZ group on the 4th and 5th days (+ p <0.05, +++ p <0.001).

Figure 1 shows the times of experimental groups to find the average platform by days.



Average time to find the platform in days of all three groups [STZ: Streptozotocin; STZ + Mel: Streptozotocin + Melatonin; The time to find the platform in the STZ group was longer on the 3rd, 4th, and 5th days compared to the control (* p <0.05, *** p <0.001). STZ + Mel group found the platform shorter on the 4th and 5th days compared to the STZ group (+ p <0.05, +++ p <0.001)]

Figure 1. Water Maze Test

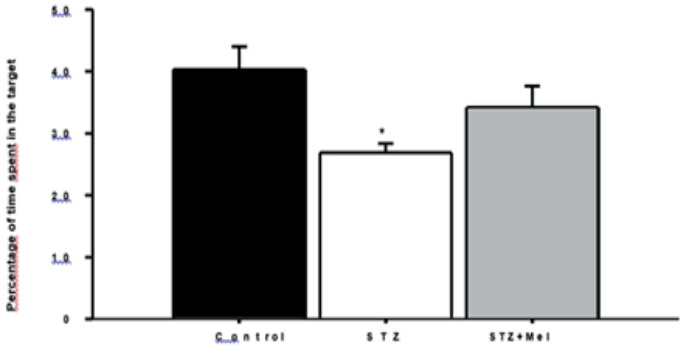


The swimming distance traveled to find the platform on the days of all three groups [The distance traveled by the STZ group to find the platform was longer on the 3rd and 5th days compared to the control (* p <0.05, ** p <0.001). The distance traveled by the STZ + Mel group to find the platform was shorter than the STZ group on the 5th day (+ p <0.05). STZ: Streptozotocin; STZ + Mel: Streptozotocin + Melatonin]

Figure 2. Water Maze Test

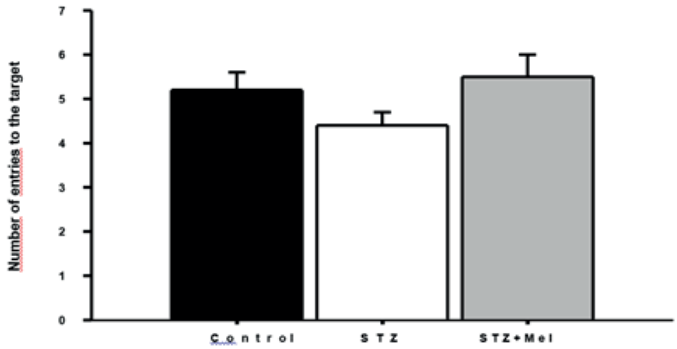
It was observed that the average swimming distance of diabetes, diabetes + melatonin, and the control group to find the platform gradually decreased from day 1 to day 5. Diabetic mother's offspring (STZ group rats) swam longer than the control to find the platform (the longer the distance means the worse learning). The difference between the STZ and the control group was statistically significant on the 3rd and 5th days (p <0.05, p <0.001). STZ + Melatonin group rats had a statistically significant difference on the 5th day to find the platform compared to the STZ group, and they had a shorter distance (+ p <0.05). Figure 2 shows the distance taken by the groups to find the platform according to days.

Probe test is an experiment that tests memory consolidation. The time spent by the subject in the quadrant where the platform was located is measured as a percentage. Diabetic mother's offspring (STZ group rats) had less time duration in the quadrant where the platform lifted during the probe test was previously localized compared to the control group (having less time means that memory enhancement was poor p <0.05). Although the difference was not statistically significant, it was found that STZ + Melatonin group was spent be a longer time in the target quadrant than the STZ group. This means that melatonin partially corrects memory impairment. Figure 3 shows the percentage of time the experimental groups spent in the quadrant with the platform.



The percentage of time that all three groups initially spent in the target quadrant where the platform was localized [STZ group remained less in the target quadrant than the control (* p <0.05). The STZ + Mel group remained more in the target quadrant than the STZ group but was not statistically significant. STZ: Streptozotocin; STZ + Mel: Streptozotocin + Melatonin]

Figure 3. Probe Test

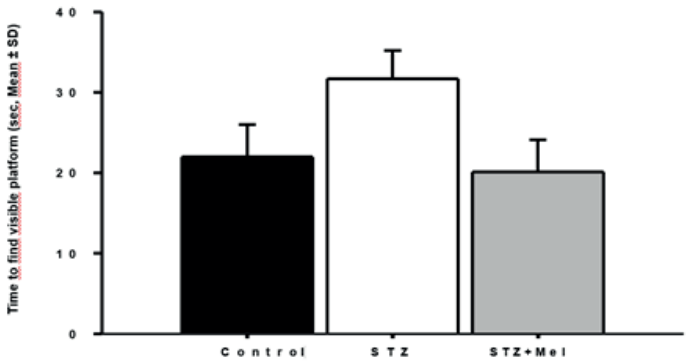


There was no significant difference between the groups; STZ: Streptozotocin; STZ + Mel: Streptozotocin + Melatonin

Figure 4. Number of entries to the target quadrant of all three groups

The number of entries of the experimental groups to the quadrant in which the platform is localized at the beginning was also evaluated, but no significant difference was observed between the groups (Figure 4).

Visual testing is an experiment that tests whether there are visual, physical, or sensorimotor defects. In the visual test performed after making the platform visible, it is seen that there is no significant difference between the times to find the platform of all three groups (Figure 5). This means that the rats in all three groups did not have visual, physical, or sensorimotor defects.



There was no significant difference between groups. STZ: Streptozotocin; STZ + Mel: Streptozotocin + Melatonin

Figure 5. Time for subjects to find the platform in visual test

NCAM Levels in Neonatal Rat Total Brain

In all three groups, the level of NCAM 180 isoforms in the total newborn brain was higher than the other isoforms. The expression of the 180 kDa isoform of NCAM in the juvenile total brain of the STZ group was found to be significantly lower than the control ($p < 0.05$). It was observed that NCAM 180 expression increased significantly in the STZ + Melatonin group compared to the STZ group ($p < 0.05$).

Expression of the 180 kDa isoform of NCAM in the STZ group was significantly lower than the control ($p < 0.05$). NCAM 180 expression increased in STZ + Melatonin group compared to STZ group ($p < 0.05$).

GFAP Levels in Neonatal Rat Total Brain

In diabetic baby rats (STZ group), the 49 kDa core band of GFAP was found to be significantly lower in the total brain compared to the control ($p < 0.001$). It was observed that GFAP expression increased significantly in the STZ + Melatonin group compared to the STZ group ($p < 0.01$).

The 49 kDa band of GFAP in the STZ group is significantly lower than the control ($p < 0.001$). GFAP expression increased significantly in STZ + Melatonin group compared to STZ group ($p < 0.01$).

Glutathione (GSH) Levels in Neonatal Rat Total Brain

GSH levels were measured in the total brains of newborn rats in the experimental groups. GSH levels were found to be significantly decreased in newborn rats in the STZ group compared to the control group ($p < 0.05$). Although it was not statistically significant, an increase was observed in the total amount of GSH in the STZ + Melatonin group compared to the STZ group.

GSH level in the STZ group is lower than control $p < 0.05$. GSH level of STZ + Mel group is higher than the STZ group; but it was not statistically significant ($p > 0.05$).

Lipid Peroxidation (MDA + 4-HDA) Levels in Newborn Rat Total Brain

In experimental groups, LPO (MDA + 4- HDA) levels were measured in the total brains of newborn rats. LPO levels in total brains of newborn rats in the STZ group were found to be significantly higher than the control group ($p < 0.001$).

LPO levels in STZ + Mel group were significantly lower than in STZ group ($p < 0.01$). This shows that melatonin administration reduces the increased lipid peroxidation in the STZ group.

LPO level in STZ group was significantly higher than control group ($p < 0.001$). On the other hand, LPO level in STZ + Mel group was significantly lower than the STZ group ($p < 0.01$).

DISCUSSION

STZ-induced diabetes is an empirically good model. In STZ-induced diabetes, chronic oxidative stress occurs as a result of hyperglycemia. Effects of oxidative stress and antioxidants on neuron damage have been studied in diabetic patients and diabetes experiments. Diabetes-related hyperglycemia generates reactive oxygen formation and RNOS, which in turn initiates lipid peroxidation of the cell membrane, damages DNA, thus increasing neuronal death by oxidant proteins (10). Human studies conducted to evaluate the intelligence and neurological functions of children of diabetic mothers often yield conflicting results. In most of the available studies; even though it was concluded that intelligence and behavioral function correlated directly with the degree of maternal glycemic control in the children of diabetic mothers (11,12), it was reported that there was no difference in memory and behavior between the children of diabetic mothers and the control group in a few studies (13). Some publications show that there is no difference in intelligence between the children of diabetic mothers and the control group and that motor disorders, distractibility, and hyperactivity are responsible for the situation that causes this slowdown in intelligence tests (14). It was observed that there was a delay in neurological development in 3.9 to 37% of the children of diabetic mothers studied (15). In diabetic patients, moderate cerebral atrophy, an increase in brainstem lesions, and subcortical lesions have been reported (16). In adults, moderate learning and memory impairment is seen along with DM (17). In diabetic patients, the development of cerebral disorder can be delayed by complete glycemic control (18). However, it is not clear whether the already occurred changes can be reversed. A series of central nervous system anomalies have been reported in the follow-up of children exposed to maternal diabetes in intrauterine life. These anomalies include weakened motor function, low intelligence level, Erb's palsy, paralysis, cerebral palsy, mental retardation, speech disorder, reading difficulties, behavioral disorders, deafness, and psychosis (19). In a study conducted on

mice with genetic diabetes, growth retardation was found in the myelin sheath and neural membranes, together with low brain weight in offspring (20). Oxidative stress is considered as an underlying mechanism for diabetic complications and diabetes. Free radicals are constantly produced in the body as a result of interaction with environmental stimuli and normal metabolic processes. Under physiological conditions, most of the antioxidants protect the body against the negative effects of free radical production in the living environment. Oxidative stress is caused by an imbalance between radical generation and the radical scavenger system. For example; oxidative stress may occur in both increased free radical production or decreased antioxidant activity. One of the free radical groups that cause oxidative stress is ROS. ROS is elevated in people with diabetes. ROS can directly destroy neurons and Schwann cells in peripheral nerves and compromise antioxidant protection mechanisms in diabetic patients (21). It has been reported that antioxidants should be used to reduce cognitive function impairment associated with diabetes and to prevent neurodegeneration. In current experiments, it has been observed that melatonin improves the impairment of cognitive functions in diabetic rats via increasing glutathione levels by decreasing lipid peroxidation. However, the mechanism of this positive effect is not fully known (10).

In our study, we observed that the learning of rats whose mothers had diabetes was at a lower level compared to the control group rats and that melatonin administration during their pregnancy positively affected the learning in diabetic rat pups. In previous studies, a learning deficit was detected in female rats whose mothers had diabetes, while no difference was found in male rats (5). We conducted our study on male rats whose mothers were diabetes, and we found that male rats also had a learning deficit. This difference may be due to the use of rats of different species in studies. In our study, we found a similar performance of all three groups of rats on a visible platform. These findings showed that the effect of diabetes on the impaired performance of infant rats was due to cognitive impairments rather than sensorimotor deficits. In studies conducted with the same type of diabetic rats, learning disability was detected, as in our study (22). In an in vitro study; It has been observed that the increased amount of extracellular glucose in rats has an inhibitory effect on the growth of neural crest cells. By examining the neural crest cells taken from embryos of diabetic mothers, it was shown that cell migration was reduced at all glucose levels and there was a decrease in migration ability in cultures formed at basal glucose concentrations. As a result of these findings, it has been reported that diabetes affects the development of premigratory cranial neural cells with a continuous effect (23). While Kinney et al. detected hyperactivity in male offspring of diabetic rats in the elevated plus-maze test, they did not find such a pattern in females. The same researchers also performed the evaluation of learning and memory in diabetic mothers' offspring in Spraque-Dawley rats. In the Lashley III Maze test, learning deficits were more prominent in female offspring belonging to diabetic mothers, but no difference was found in male rats. The memory test was performed 2 and 4 weeks after the learning test, and in the test at the end of the second week, it was found that female offspring of diabetic mothers made significantly more errors, while the result of the same test was not significant in males. There was no significant difference in the number of mistakes made by males and females in the test in the 4th week. In the study investigating the effects of exposure to hyperglycemia in neonatal life on short-term (immediate) memory, it was observed that control and diabetic rat pups learned the given task. There was no difference between the female offsprings and the control group and between the males and the control group (5).

In our study, we observed that male rats whose mothers had diabetes had worse memory than the control group and that melatonin administration during pregnancy partially corrected the memory impairment in diabetic rat pups.

In the study conducted to examine the learning-inhibiting effect of maternal hyperglycemia, the diabetic mother received 5 IU/kg/day insulin support during lactation and fed her offspring. When the median step-through latency of the offspring of the rats in diabetes and control groups were compared, no difference was observed. The median step-through latency of daughters belonging to diabetic mothers during the memory study was found to be significantly shorter than the control group. No significant difference was found between control and male offspring (5). In their study, Kinney et al observed that learning disorders were detected in especially female offspring of the diabetic mother rather than male offsprings.

In short-term memory and immediate memory studies, there was no difference in leukomotor activity and motivation between the offspring of diabetic mothers and the control group, and the near-term memory results were also found to be the same. It was thought that the changes in the mental development of the diabetic female rat pups affected certain regions of the brain, especially a number of centers associated with long-term memory, rather than an effect on the whole brain. The observation that learning differs with gender in offspring of diabetic mothers suggests that diabetes in the intrauterine environment may affect different mental development focuses in children depending on gender (5). It is suggested that NCAM causes the formation of synaptic changes during learning and memory formation (24).

In our study, we looked at NCAM and GFAP molecules in the brain tissue of juvenile rats to investigate the link between learning and memory impairments in hippocampal synaptic plasticity and neurogenesis in rats whose mothers had diabetes. Our study investigated the changes in GFAP and NCAM in the brain tissue of offspring whose mothers have diabetes, their relationship with learning, and the protective effect of melatonin. In DM, hyperglycemia can prevent synaptic rearrangement between neurons. Also, PSA decreases in diabetes. Thus, instead of the negative correlation between NCAM 180 level and learning, the

imbalance in the NCAM level, the change in the PSA level and/or the communication disturbance between the two decreased synaptic plasticity, the underlying mechanism of memory, and learning function (25). Studies of diabetic rats with NCAM deficiency show learning impairment. These findings may indicate that NCAM has a role in learning and long-term memory formation. Diabetes may interfere with the formation of synaptic plasticity and learning by inhibiting the polysialization of NCAM in the brain tissue of the offspring. Brain development and synaptic plasticity formation may be inhibited in diabetic rat pups as a result of NCAM deficiency (22). It is suggested that NCAM 180 is associated with cognitive functions. It has been suggested that NCAM 180 is an important determinant of synaptic plasticity and affects the stabilization of synaptic power (26). The decrease in NCAM 180 expression causes synaptic destabilization related to the storage of information (27). In our study, the level of NCAM 180 isoforms in the total brains of newborn rats in all three groups was found to be higher than the other isoforms. Expression of the 180 kDa isoform of NCAM in the offspring total brain in the diabetes group was found to be significantly lower than the control. This result may be the cause of learning impairment. It was observed that NCAM 180 expression of the rat pups in the group treated with melatonin during pregnancy significantly increased compared to the diabetic group. Most studies conducted in recent years show the role of NCAM in determining learning and long-term memory (28,29). Comparing diabetic animals with non-diabetic animals in terms of adaptability to learning, the level of NCAM in diabetics was found to be decreased. With the intracranial injection of NCAM antibodies, it has been observed that antibodies help inhibition in the passive avoidance task (30). In diabetic rats, upregulation of NCAM in the hippocampus and cortex plays a potential role in regulating tissue reorganization. When diabetic rats and control groups were compared, it was observed that the amount of NCAM 180 increased excessively in the hippocampus and cortex of diabetic rats (22). In our study, the level of NCAM 180 isoforms in the total neonatal brain in all three groups was found to be higher than the other isoforms. NCAM 180 is the main form of NCAM and is required for cell attachment stabilization in synaptic regions (31). NCAM 180 is known to affect the spectrin and its cytoplasmic part.

GFAP is the major intermediate filament of mature astrocytes. One of the key events during astrocyte differentiation is increased GFAP expression. Immature astrocytes initially express vimentin and then GFAP at maturity. GFAP is recognized as an astrocyte maturation marker. While GFAP level is extremely low in fetal life, its level increases with the development of the brain (27). In our study, the 49 kDa core band of GFAP was found to be significantly lower in rats whose mothers were diabetic compared to the control. It was observed that GFAP expression increased significantly in the melatonin group during pregnancy compared to the diabetes group.

In diabetes, protein glycation and glucose autoxidation can produce free radicals that subsequently catalyze LPO (32,33). Besides, it has been shown that the antioxidant defense system is impaired in diabetes; changes in antioxidant enzymes, impaired GSH mechanism, and decreased ascorbic acid levels are seen (34,35). However, high oxidative stress in a living environment has never been clearly demonstrated. Lipoproteins and increased LPO in membranes in diabetic structure have been demonstrated in studies performed in animal and human models using thiobarbituric acid analysis substance (36). Having a free sulfhydryl group, reduced GSH is effective as an intracellular sulfhydryl buffer and protects cells against oxidative and toxic effects. Products released as a result of LPO significantly affect membrane permeability and microviscosity. Chronic hyperglycemia accompanies increased oxidative stress markers such as LPO, protein oxidation, and deoxyribonucleic acid oxidation (10). In studies, LPO levels were found to be high in various brain regions of diabetic rats, GSH levels were found to be low, and in diabetic rats treated with vitamin E, melatonin, and gabapentin, LPO levels were found to be lower and GSH levels were higher than in the untreated diabetic group (10,37). In the Morris Water Maze test, the learning of rats with high LPO and low GSH levels was found to be impaired than the control group (10). These findings may suggest that increased oxidative stress may affect learning and memory.

In our study, we found higher LPO levels in the diabetic group compared to the control group. The increased lipid peroxidation in the diabetes group decreased with melatonin administration. GSH levels were found to be lower in the diabetic group compared to the control group. With the administration of melatonin, a partial increase was observed in GSH levels in the diabetic group. This may show us that the antioxidant treatment that mothers with diabetes will take during their pregnancy may protect their offspring against the harmful effects of oxidative stress.

CONCLUSION

As a result, learning and memory functions are impaired in the offspring of diabetic mothers. Decreasing NCAM isoforms can inhibit brain development and synaptic plasticity formation and may indicate the role of NCAM in the formation of learning and long-term memory. Decreasing GFAP density can cause a problem in completing brain maturation in offspring of diabetic mothers. Free oxygen radicals occurring in diabetes cause neuronal death by the effects of peroxidation of membrane lipids, DNA damage, and protein oxidation. In diabetes, brain development and synaptic plasticity formation can be regulated by blood sugar regulation during pregnancy, and learning deficiency and delay in brain maturation can be prevented.

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