PHARMACOLOGY

EFFECTS OF VITAMIN D₃ AND RESVERATROL ON LEARNING AND MEMORY DISABILITIES OF STREPTOZOTOCIN-INDUCED DIABETIC MICE

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Abstract: This study was conducted for investigating the effects of chronically administered vitamin D₃ and resveratrol, which has antioxidant properties, on memory and learning impairment caused by streptozotocin-induced diabetes. Saline was administered to the mice in the first group. Streptozotocin (60 mg/kg, i.p.) was given to the mice in the second group for 3 consecutive days and no treatment was performed. Vitamin D₃ (800 IU/kg, i.p.) was given to the streptozotocin-induced hyperglycemic mice in the third group for 4 weeks. Resveratrol (40 mg/kg, i.p.) was given to the streptozotocin-induced hyperglycemic mice in the fourth group for 4 weeks. Then, the passive avoidance test was applied to the mice. A significant difference was found between DM+Vit D₃ group (21.6 ± 1.7, n = 7) and diabetic control group (28 ± 2.6, n = 7) in the acquisition test ($p < 0.001$). A similar result was observed between diabetic-resveratrol group (23.7 ± 2.5, n = 7) and the untreated diabetic mice group (28 ± 2.6, n = 7). There was a significant difference. 4-week vitamin D₃ (400 IU/day, i.p.) and 4-week resveratrol (40 mg/kg, i.p.) treatments given to streptozotocin-induced diabetic mice were observed to significantly improved the passive avoidance acquisition and retention times. These results show the positive effects of vitamin D₃ on memory.

Keywords: diabetes, memory, vitamin D₃, resveratrol, learning

Diabetes mellitus is a metabolic disease that results from lack of insulin hormone secreted from the pancreas or by increasing amount of sugar in blood due to the resistance in tissues caused by insulin (1). It affects negatively brain and other vital organs. Important health problems such as cardiovascular diseases, renal failure and eye diseases may occur in the future depending on consistently high blood sugar (1). Diabetes affects the blood vessels in the brain and the body and it thus may cause memory loss (2). Diabetes should be monitored on time otherwise it may damage learning and memory (3). Diabetic patients are under the risk of memory problems in case they could not control blood sugar (3). Diabetes has a significant role in memory and learning as well as insulin and its receptors have a significant role in nervous system diseases such as Alzheimer (2, 4).

Diabetes mellitus is the main reason why chronic inflammation occurs in the brain for elderly people. This inflammation affects the blood vessels and weakens the brain tissues over time. It also affects the gray matter that is associated with decision making and reasoning abilities and it thus makes the patient unable to perform his/her easy tasks (2, 5).

Vitamin D is a steroid hormone that affects all cells so it is crucial to keep it at healthy levels. Low levels of vitamin D damage the bones. It is also far more important for the heart, brain and immune system functions. For example, there is a crucial correlation between vitamin D deficiency and insulin resistance or type 1 and type 2 diabetes (2, 4, and 6). Vitamin D may affect the risk of diabetes type 2. According to recent studies, vitamin D deficiency affects glucose metabolism and actually causes diabetes rather than obesity (2, 4). Studies on the animals have shown that vitamin D is one of the key factors in the normal secretion of insulin and it also improves insulin sensitivity (5, 6). The aging process is a reason for establishing a correlation between vitamin D and the nervous system (6). Vitamin D deficiency may cause bone thinning, fall or fracture risk in the elderly (7). And it causes cancer, coronary artery disease and an increase in mortality (8). For these reasons, it is considered that vitamin D and aging is associated with (9). Memory loss and absence of perception occur with aging (9).

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The memory loss and absence of perception arising in the aging process are caused by degeneration of the nervous system depending on diseases such as Parkinson and Alzheimer diseases. It may be also considered that Vitamin D may be associated with cerebral problems if it is associated with diseases arising in the old age.

People affected by diabetes are exposed to early arterial aging (10). Sclerosed arteries increase the risk of heart attack, paralysis and high blood pressure (10). Previous studies using animals as experimental subjects have shown that Resveratrol helps to reduce aortic sclerosis (11). Resveratrol treatment can reverse the abnormalities in the blood vessels caused by aging, obesity and diabetes (11). It means that resveratrol treatment is beneficial for diabetic patients.

This study conducted on experimental animals has been planned in order to examine the use of vitamin D and resveratrol in diabetic patients.

MATERIAL AND METHOD

Animals

Experimental animals were supplied by Ondokuz Mayıs University Experimental Animal Research and Application Center. 28 BALB/c male mice (30-40 grams) of which weights were very close to each other were selected for the experiment. The animals were kept and fed in the pharmacology laboratory at the normal room temperature (22°C) for a week before the experiment so that mice could adapt to the environment. Animal experiments were conducted in accordance with national guidelines on the use and care of laboratory animals. Approvals were obtained from Ondokuz Mayıs University Clinical Research Ethics Committee (No: 2018/34).

Chemical substances

The vitamin D_3 used in the experiment was supplied by Deva Holding Co. Turkey and streptozotocin and resveratrol was supplied by SIGMA-USA.

Inducing hyperglycemia

Hyperglycemia in mice was induced by streptozotocin. The streptozotocin dissolved in distilled water was intraperitoneally injected to the mice in 60 mg/kg dose for three days in a row. Then, preprandial blood glucose was measured in blood samples from the tail veins of mice three days after streptozotocin had been administered. A commercially available blood measuring device was used to measure the blood glucose. Animals with a 250

mg/dL blood glucose level and above were involved in our study. As is known, those who are with a blood glucose level higher than 250 mg/dL are regarded as diabetic (12).

Experimental groups

In the experiments, mice were classified into 4 groups and each group included 7 mice. Twenty-eight albino Balb/C mice (white mouse) were used. The drugs were injected intraperitoneally (i.p.).
1. Non-diabetic (No treatment group)
2. Untreated diabetic group administered with streptozotocin (control)
3. Diabetic treatment group administered with streptozotocin + vitamin D_3
4. Diabetic treatment group administered with Streptozotocin + Resveratrol

Experimental procedure

Physiological saline solution was administered to the mice in the first group (n = 7). Streptozotocin (60 mg/kg, i.p.) was administered to the mice in the second group (n = 7) for 3 days in a row and no treatment was not administered. Vitamin D_3 (800 IU/kg, i.p.) was administered to the streptozotocin-induced hyperglycemic mice in the third group (n = 7) for 4 weeks. Resveratrol (40 mg/kg, i.p.) was administered to the streptozotocin-induced hyperglycemic mice in the fourth group (n = 7) for 4 weeks. Drugs were injected intraperitoneally half an hour (retention period) before the test. Physiological saline solution was administered to the mice in the control group. Then, the mice were subjected to the passive avoidance test. Passive avoidance instrument (Ugo Basile, Passive Avoidance Controller Cat 7551, Italy) was used in the passive avoidance test. This instrument has two identical cells in 21 × 22 × 21 cm. One of the cells is made of completely dark color Plexiglas, and the other one is made of white, opaque, bright Plexiglas. The bright cell is illuminated by a light source on the top.

Once the mice pass from bright cell to the dark cell, a 0.5 mA electric shock is supplied for 3 seconds and time to pass from light cell to the dark one is recorded. The door between the cells is closed automatically to prevent mice from moving back to the bright area after they pass to the dark cell and electric shock is supplied on their feet. Therefore, the mouse stays in the dark cell after the electric shock is disconnected.

The passive avoidance test was conducted in two steps. The first step is a two-step period including pre-acquisition/pre-learning period and acquisition/learning periods. As for the second step, it is

retention period which is assumed that knowledge acquired and thought to be kept in the acquisition/learning period one day ago would be remembered after 24 h. Experimental subjects were subjected to pre-acquisition period on the experiment day before acquisition period that is the first step of the test in order to make subjects get used to the environment (pre-acquisition/pre-learning period). In the pre-acquisition phase, experimental subjects were put in a bright cell of passive avoidance instrument and subjected to the test. The mice put in the bright cell one by one usually moves to the dark cell in 60 s. The mice failed to move to the dark cell in 60 s were excluded from the experiment. Any electric shock wasn't supplied at this stage.

The mice put in the acquisition period and moved to the dark cell in the same day were exposed to electric shock for a specified time and current (0.5 mA, 3 s) through the wire grids on the surface. Then, the mice exposed to electric shock were taken from this cell and put back in their cages. The mice are supposed to acquire/learn the knowledge of being exposed to electric shock in this period and store it to remember one day later. The second stage of the test was performed after 24 h. The mice exposed to electric shock one day ago were placed into a bright cell of the instrument after 24 h in the same order and in the same way. Any electric shock wasn't supplied again to the mice moved to the dark cell. The time to move (retention period) to the dark cell (the cell where they were exposed to electric shock) was measured, and the mice moved to the dark cell were put back into their cages. In this test, it is considered that it is an indicator that experimental subjects have

learned if they do not move to the dark cell one day later where they were exposed to electric shock one day ago or they do not move to the dark cell at any time (compared to the previous day). And it is accepted that learning is affected negatively or disturbed if the experimental subjects move to the dark cell more quickly compared to the previous day.

Statistical analysis

Data is analyzed using one-way ANOVA and post-hoc Tukey tests. Data is expressed as mean value ± standard deviation and significance level is accepted as $p < 0.05$.

RESULTS

Figure 1 shows the time to enter the dark cell (initial latency). The figure illustrates non-diabetic mice, diabetic-untreated mice, diabetic + vitamin D_3 administered mice, and diabetic- resveratrol administered mice, respectively. A significant difference was found between DM + Vit D_3 group (21.6 ± 1.7, n = 7) and diabetic control group (28 ± 2.6, n = 7) in the acquisition test ($p < 0.001$). There was a significant difference between non-diabetic mice group (22.6 ± 3.31, n = 7) and diabetic-untreated mice group (28 ± 2.6, n = 7) ($p < 0.005$). A similar result was observed between diabetic-resveratrol group (23.7 ± 2.5, n = 7) and the diabetic-untreated mice group (28 ± 2.6, n = 7). A significant difference was found. These results, namely obtaining a significant difference between streptozotocin-induced diabetic mice and diabetic- vitamin D_3 administered mice, shows us the positive effects of vitamin D_3 on learn-
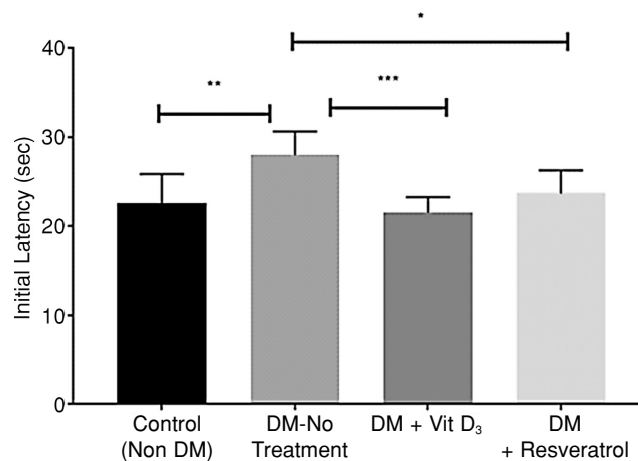

Figure 1. Times to enter into the dark compartment of the non-diabetic, diabetic-untreated, diabetic + vitamin D_3 administered, diabetic + resveratrol-administered mice groups during the pre-acquisition phase of the passive avoidance test (initial latency). The data were expressed as mean ± standard deviation (n = 7). *p < 0.05, **p < 0.01, ***p < 0.001

ing and memory. Similarly, obtaining a significant difference between streptozotocin-induced diabetic mice and diabetic-resveratrol administered mice shows us resveratrol's positive effect on memory and learning. In the acquisition period of the passive avoidance test, however, any significant difference wasn't observed between non-diabetic mice control group and diabetic- vitamin D_3 administered mice group ($p > 0.05$). Similarly, any significant difference wasn't observed between the non-diabetic mice

control group and diabetic- resveratrol-administered mice group ($p > 0.05$).

The retention tests constituting the second part of the passive-avoidance test were performed after 24 h (Fig. 2). A significant difference was observed between diabetic mice group (111.2 ± 5.6, n = 7) and diabetic-vitamin D_3 administered mice group (141 ± 12.3, n = 7) ($p < 0.005$). A significant difference was observed also between diabetic mice group (111.2 ± 5.6, n = 7) and diabetic-resveratrol administered
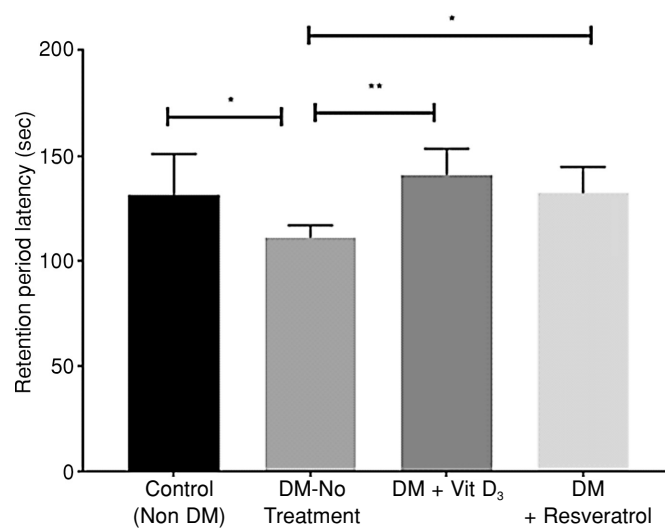

Figure 2. Times to enter into the dark compartment of the non-diabetic, diabetic-untreated, diabetic + vitamin D_3 administered, diabetic + resveratrol-administered mice groups during the acquisition phase of the passive avoidance test (Retention period latency). The data were expressed by the mean $\pm$ standard deviation (n = 7). *$p < 0.05$, **$p < 0.01$
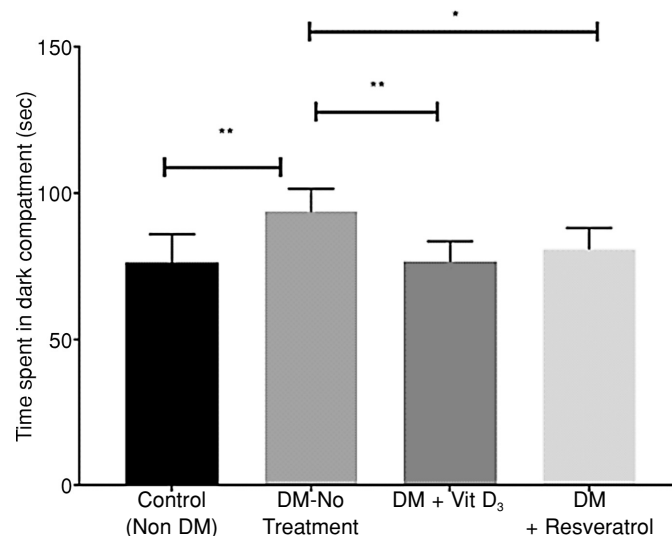

Figure 3. Time spent in the dark compartment of the non-diabetic, diabetic-untreated, diabetic + vitamin D_3 administered, diabetic + resveratrol-administered mice groups during the passive avoidance test acquisition period. The data were expressed by mean$\pm$standard deviation (n = 7). *$p < 0.05$, **$p < 0.01$

mice group (132.2 ± 12.5, n = 7) (p < 0.05). In addition, a significant difference was observed also between non-diabetic mice control group (131.5 ± 19.4, n = 7) and diabetic-untreated mice group (111.2 ± 5.6, n = 7) (p < 0.05). Any significant difference wasn't observed between the non-diabetic mice control group and diabetic-vitamin D_3 administered mice group (p > 0.05). Similarly, any significant difference wasn't observed also between non-diabetic mice control group and diabetic-resveratrol-administered mice group (p > 0.05).

On the other hand, when evaluating the time spent by the mice in the dark cell of passive avoidance test, a significant difference was observed between the non-diabetic mice control group (76.1 ± 9.8, n = 7) and the diabetic-untreated mice group (93.7 ± 7.8, n = 7) (p < 0.005). A significant difference was observed between diabetic mice group (93.7 ± 7.8, n = 7) and diabetic-vitamin D_3 administered mice group (76.6 ± 6.9, n = 7) (p < 0.005). Similarly, a significant difference was observed also between diabetic mice group (93.7 ± 7.8, n = 7) and diabetic-resveratrol administered mice group (80.9 ± 7.2, n = 7) (p < 0.05). Considering the time spent in the dark cell in the passive avoidance test, any significant difference wasn't observed between the non-diabetic mice control group and diabetic-vitamin D_3 administered mice group (p > 0.05). Similarly, any significant difference wasn't observed between the non-diabetic mice control group and diabetic-resveratrol administered mice group (p > 0.05).

DISCUSSION

This study has been carried out to examine the effects of chronically administered vitamin D_3 and resveratrol having antioxidant properties on memory and its effects on learning disorder caused by streptozotocin-induced diabetes. Streptozotocin-induced diabetic mice decreased the entrance time, indicating that there is an impairment in learning and memory period. Learning and memory impairment period in streptozotocin-induced diabetic mice has been demonstrated experimentally. In this study, a significant improvement was observed in passive avoidance acquisition time and retention time for streptozotocin-induced diabetic mice subjected to 4-week vitamin D_3 (400 IU/day, i.p.) and 4-week resveratrol (40 mg/kg, i.p.) treatments.

The association of diabetes with memory and learning functions is not clear yet. The recent studies indicate that vitamin D deficiency may increase diabetes risk (13). Scientific studies draw our attention to effects of vitamin D on diabetes and glucose control (14, 15). According to recent studies, vitamin D deficiency affects glucose metabolism and actually causes diabetes rather than obesity (16, 17). Studies conducted on the animals have shown that vitamin D is a key factor for normal insulin secretion and it improves insulin sensitivity (18, 19).

These results show us the positive effects of vitamin D_3 on memory. Our results are compatible with the previous studies. The curative effects of vitamin D_3 on learning and memory may partially depend on the physiological effects of calcium homeostasis (20). Calcium has a buffering role (20). It has a role also in memory formation and synaptic plasticity (21). Our study is compatible with the antioxidant effects of vitamin D. It has been demonstrated in a clinical study by Nerhus M. et al. that vitamin D improves cognitive functions of the patients with psychotic disorder (23). In a different study, vitamin D deficiency resulted in cognitive failure for male mice (24). These results are compatible with findings obtained from our study.

Role of oxidative stress and free radicals in diabetic complications has been demonstrated (24, 25). Oxidative stress formation in diabetes depends not only on the formation of oxygen-free radicals but also on the antioxidant enzyme failure and the formation of peroxide (26).

Resveratrol is a flavonoid having antioxidant properties which have been used in the treatment of diabetes in recent years (27, 28). People affected by diabetes are subjected to early arterial aging. Sclerosed arteries increase the risk of heart attack, paralysis and high blood pressure risks. Previous studies using animals as experimental subjects have shown that resveratrol helps to reduce aortic sclerosis (29). Resveratrol treatment can reverse the abnormalities in the blood vessels caused by aging, obesity and diabetes (30). It means that resveratrol treatment is beneficial for patients with diabetes.

Healing effect of resveratrol as an antioxidant on learning and memory disabilities was demonstrated in the study by Wang R. et al. (31). Healing role of resveratrol in cognitive failure induced by chronic stress was demonstrated in another study by Shen J. et al. (32). In a study by Tian Z. et al., cognitive failure was healed as resveratrol had regulated apoptosis and synaptic plasticity (33). These findings concerning the effects of resveratrol on learning and memory are compatible with also our study.

The antioxidant defense mechanisms of the brain are weaker and therefore the brain is more sensitive to oxidative stress. Any increase in oxidative stress may disturb memory (34). Certain studies have also shown that vitamin D_3 treatment has a role

in regulating blood glucose and has antioxidant activity (35-37). Vitamin D_3 may result in an improvement for brain functions by activating the antioxidant system (36, 37). In conclusion, we can say depending on the latest studies we have that vitamin D_3 has a healing effect in the pre-acquisition and retention period of the passive avoidance test. In conclusion, we need further comparative studies in order to obtain more data concerning cognitive functions.

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