Vitamin D mitigates hippocampus apoptosis induced by diabetes

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Abstract:

Background: Diabetes mellitus (DM) is a chronic progressive metabolic illness and that is greatly associated with an increased risk of developing cognitive deficits. Numerous previous studies have indicated that vitamin D has neuroprotective effects. However, the impact of vitamin D on type 2 diabetes mellitus (T2DM)-associated hippocampal damage in the brain and its protective mechanism remains unclear.

Objectives: We investigated the effect of vitamin D supplementation on diabetes-related apoptotic changes in the hippocampus of diabetic rats.

Materials and methods: Our study is an experimental randomized control trial conducted between November 2020 to March 2021in the Physiology Department, Faculty of Medicine, South valley university. We constructed a T2DM rat model on 24 Male albino Sprague Dowely rats; diabetes was induced by a high- fat diet and a single low dose of streptozotocin (STZ) (30mg/kg). Animals were divided into four groups; Normal control, Control receiving vitamin D (VD), Diabetic control, and Diabetic receiving vitamin D group.

Results: Compared with the control, results showed decreased spontaneous alternation T maze cognitive test, declined neural survival and increased immunohistochemistry expression of Synaptophysin in the hippocampus in diabetic rats. Vitamin D supplementation for six weeks can ameliorate diabetes-associated cognitive impairments by increasing neural cell survival and reducing neural apoptosis in the hippocampus.

Conclusion: The resulting data have the potential to provide vitamin D as a new type of adjuvant agent for anti-diabetic lines of treatment.

Keywords: T2DM; Apoptosis; Vitamin D; Synaptophysin; Cognition

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Introduction

Diabetes mellitus is a common metabolic disease around the world. More than 90% of diabetic patients have T2DM. the micro and macrovascular problems of diabetes are extremely distressing for both diabetic patients and caregivers **(Chatterjee et al., 2017; Mohamed et al., 2021)**.

T2DM leads to many chronic complications, including peripheral neuropathy, diabetic retinopathy, and cardiovascular disease. **(Bellia et al., 2022)**. Diabetes mellitus, both type 1 and type 2, has been linked to diminished cognitive function. Diabetes can cause cognitive deficits even in the very early stages of diabetes, cognitive impairments may exist. The kind and severity of cognitive impairment may be influenced by the duration of diabetes and glycemic control **(Zilliox et al., 2016)**.

People with T2DM have a risk of poor cognitive performance associated with memory loss and executive dysfunction. Although the primary goal in the management of diabetes is glycemic control, current anti-diabetic medications have neuroprotective effects that need to be given serious consideration**(Pugazhenthi et al., 2017; Alkethiri et al., 2021)**.

One of the most vulnerable regions in brain to the metabolic disease including diabetes mellitus is the Hippocampus **(Foghi and Ahmadpour, 2014)**. The hippocampus is a paired and horse shoe- like structure, with one hippocampus located in the left brain hemisphere and the other in the right hemisphere **(Squire 1992)**. The hippocampus is a corner part of the limbic system, it is crucial for memory formation and emotional, reproductive, and adaptive behaviors **(Squire 1992),** and also has an important role in forming new memories, the hippocampal structural damage mainly affects recently acquired memories, however previously acquired old memories remain unaffected **(Kitamura and Inokuchi , 2014)**.

Studies have demonstrated that cell proliferation continues in the hippocampus constantly. This unique hippocampal production of neurons in the adult brain is necessary for memory formation **(Koehl and Abrous 2011, Kitamura and Inokuchi, 2014)**.

Apoptosis is suggested to be a possible mechanism for diabetes-induced hippocampal neuronal cell death. It has been reported that apoptosis is involved in neurodegenerative disorders like Alzheimer's disease **(Li et al., 2002; Sadegh et al., 2016)**. It's reported that type 1 and type 2 diabetes have a hurt impact on impaired neurogenesis, dendritic remodeling, and increased apoptosis in the hippocampus **(Ho et al., 2013)**.

Synaptophysin is an integral membrane component of synaptic vesicles **(Arthur and Stowell , 2007)**. Numerous data show that synaptophysin is implicated in many aspects of ex-endocytosis of synaptic vesicles. Interaction between Synaptophysin and Synaptobrevin to modulate synaptobrevin's interaction with the plasma membrane–associated proteins syntaxin and SNAP-25 to form SNARE complex required for membrane fusion of synaptic vesicles **(Mitter et al., 2003; Reisinger et al., 2004)**. Synaptophysin is also implicated in synapse formation and the formation and recycling of synaptic vesicles **(Sun et al., 2006)**. Because Synaptophysin is widely distributed in all major brain areas., being used as a sensitive marker of synaptic density **(Jung et al., 2009).**

Vitamin D is well recognized as a steroid hormone for calcium metabolism and bone health; in addition, it can also be implicated in brain functions, because it passes through the blood-brain barrier. It has been demonstrated that the VD receptors and enzymes involved in its function are widely distributed in the brain **(Garcio et al., 2002)**.

Previous studies reported that patients with type 2 diabetes who have decreased serum levels of VD have mild cognitive impairment **(Chen et al., 2014)**. The possible protective effect of vitamin D3 supplementation on cognitive function is mediated via increasing cholinergic

transmission in the prefrontal cortex in diabetic animals **(Alrefaie and Alhayani, 2015).** These results highlight the necessity for continued investigation into another potential protective function of VD supplementation in the development of neurodegenerative alterations in type 2 diabetics.

In T2D patients who are VD-deficient, VD supplementation may improve T2D by lowering HbA1c and increasing SIRT1 and irisin levels **(Safarpour et al., 2020)**. Another study found that VD restored cognitive deficits caused by a high-fat diet (HFD) through increasing brain derived neurotrophic factor (BDNF) in the hippocampus and decreasing nuclear factor kappa light chain enhancer of activated B cells (NF-B) concentrations **(Hajiluia et al., 2017)**. However, the protective role of VD supplementation in neurodegenerative changes of diabetes mellitus is still debatabl although, more research is necessary because there are so few studies in this field. In the present study, we designed new therapeutic approaches for ameliorating. T2DM-associated hippocampal damage in the brain.

Materials and Methods

Our study is an experimental randomized control trial conducted between November 2020 to March 2021 in the Physiology Department, Faculty of Medicine, South Valley University. All experimental procedures were approved by the Animal Ethical Committee of the Faculty of Medicine, south valley university.

Ethical approval code: SVU-MED-PHY003 -2-21-9-2424.

Experimental animal

The study included twenty-four male albino Sprague Dowely rats, 2 months age old and weighing 160±10 g. The rat were obtained from the Egyptian Campany for Production of Antisera, Vaccines, and Drugs Hewan, Egypt. Rat were housed in polypropylene cages with stainless steel covers, three per cage. Animals were kept in a room with a 12/12-hour day/night cycle, a relative humidity, and a 22±2°C room temperature . Animals were housed for one week for acclimatization, during which they were given free access to water and the standard commercial pellet diet.

Induction of T2DM

Excluding the normal control and healthy control-treated groups, all experimental rats were given free access to a high-fat diet (40%) for a-10 weeks duration. The High fat diet consisted of 20% coconut oil and 80% standard commercial pellet diet. A digital scale was used to assess body weight once every week. After 10 weeks, the rat fasted for 6 hours before a single intraperitoneal injection with a low dose of (30 mg/kg) STZ (Catalogue No. S0130, Sigma-Aldrich Corp., St. Louis, MO, USA) dissolved in 1ml of 0.1M citrate buffer with pH 4.5M citrate buffer. Induction of T2DM was performed with guidance of **Gheibi et al., (2017)**. Diabetic animals were confirmed by blood glucose levels above 250 mg/dl 72h following injection. Diabetic rats were allowed to feed HFD continuously during the study.

Experimental design

Animals were randomly divided into four groups, six animals per group according to the following experimental design:

Group I: Normal control
Group II: Normal control receiving vitamin D
Group III: Diabetic control
Group IV: Diabetic receiving vitamin D

Vitamin D supplementation : Using the principle of the previous study of **Nadimi et al., (2020),** diabetic rats were administered vitamin D3 (cholecalciferol) by subcutaneous injection in a dose of 4300 IU/kg/week dissolved in 0.25ml of semesm oil for 6 weeks. Normal control rats and diabetic control rats received a vehicle, 0.25ml semesm oil.

Assessment of learning and memory

Before animal scarification, the learning and memory of rats were assessed by the T Maze Spontaneous Alternation test.

T Maze Spontaneous Alternation: The principle of a T-Maze using a discrete trial was performed according to the protocol of a previous study by **Grzeda et al. (2007)** . Spontaneous Alternation is based on rats' natural tendency to alternate their choice of

goal arm in a T-maze. Alternation reflects the desire of the animal to explore new surroundings. This task was performed using a T-Maze apparatus constructed from wood. The maze consisted of a long start arm (15 cm × 50 cm), at the top of which were perpendicular choice arms (10 × 50 cm). Rat was dropped into the maze at the bottom of the start arm after the maze's center partition was in place and all of its doors had been lifted. Upon leaving the start arm, the rat chooses between entering either the left or the right goal arm. The Choice was recorded once all four paws had entered the arm. Then the rat remained for 30 seconds after these goal arms were blocked. After 30s, the central partition was removed, then the animal was removed as gently as possible before the next choice run, the block was removed and the animal was placed in the start area facing away from the goal arms. The animal could then select either arm. With repeated trials, the animals should exhibit less of a tendency to enter a previously visited arm. If the animal re-entered the previously visited arm, this was an inaccurate decision. If the animal entered the alternate arm, this was the proper choice. The number of turns in each goal arm was calculated to determine the percentage of alternation.

Histopathological assays.

At the end of the experiment, animal sacrification was done by using a lethal dose of diethyl ether pure, and animals' skulls were fractured at the occipital regions using a bone cutter. On the ice, the brain was exposed and promptly dissected, and the hippocampus on both sides was quickly isolated. The hippocampus was placed in 10% paraformaldehyde for histological and immunohistochemical examination.

General histological examination: The technique for histological process is illustrated by Bancroft and Stevens (2013). In brief, the hippocampus was sliced into 3-4 µm thick, fixed with 10% neutral buffered formalin (NBF), graded ethanol concentrations for dehydration, xylene for clearing, and finally embedded in paraffin. To investigate the general tissue structure, the paraffin blocks were cut into sections using a microtome at a thickness of 4-6 µm and stained with Hematoxylin and Eosin stain(H&E).

Immunohistochemistry staining protocol: Immunohistochemistry (IHC) was carried out on paraffin sections and mounted on positively charged slides by using the avidin-biotin-peroxidase complex (ABC) method (Hsu et al., 1981). Mouse Anti-Synaptophysin Monoclonal antibody (Dako Cat# IR660, Clone: DAK-SYNAP, ready to use) (Kwon and Chapman 2011), was used in this IHC examination. Sections from each group were incubated with the previously mentioned antibody, then the reagents required for the ABC method (Vectastain ABC-HRP kit, Vector laboratories) were added. To identify antigen-antibody completion, diaminobenzidine (DAB), made by Sigma was used to a color and peroxidase to label the marker expressiox. The primary antibody was substituted with non-immune serum for the negative controls. A Leica microscope was used to view IHC stained sections. (CH9435 Hee56rbrugg) (Leica Microsystems, Switzerland) in the Faculty of Medicine, South Valley University.

Immuno-Scoring by Optical Density Measurement: Scoring was done by measurement the optical density (OD) along CA3 sections of hippocampus . Quantitative assessment was performed by screening the hippocampus sections and selecting at least six different fields in each studied group. The optical density of Synaptophysin was identified by manipulating the image analysis system of the Leica Qwin 500 (Leica Imaging Systems Ltd, Cambridge, England). The modified optical density was obtained by subtracting the original value from 255 (maximum brightness) and converting the product to logarithm.

Statistical analysis

Using SPSS version 25*, data analysis was carried out. Descriptive statistics: Means ± standard deviations (SD) were calculated. Test of significances: For continuous variables with more than two categories; ANOVA test was calculated to test the mean differences of

the data, and the post-hoc test was calculated using Tukey corrections. A p-value of 0.05 or less was regarded as significant.

Results

Effects of vitamin D supplementation on enhancing learning and memory ability in diabetic rats

A) T maze test (Spontaneous Alternation)

As described in **(Fig. 1)**, the mean value of percentage of alternation score in group II (control receiving vitamin D) is (70±20), with an insignificant difference (p =0.745) when compared with the mean value of percentage of alternation score in group I. Diabetic rats exhibited memory deficits in T-maze as compared to the control group which was evident from the significant reduction of percentage of alternation score (p =0.003) **(Fig. 1)** in T- maze from a mean value (76±15) in group I to (43±16) in group III (diabetic group). The mean value of percentage of alternation score in group IV (diabetic receiving vitamin D) is (71±13), with a significant increase (p =0.013) **(Fig. 1)** when compared with the mean value of percentage of alternation score in group III (diabetic group).

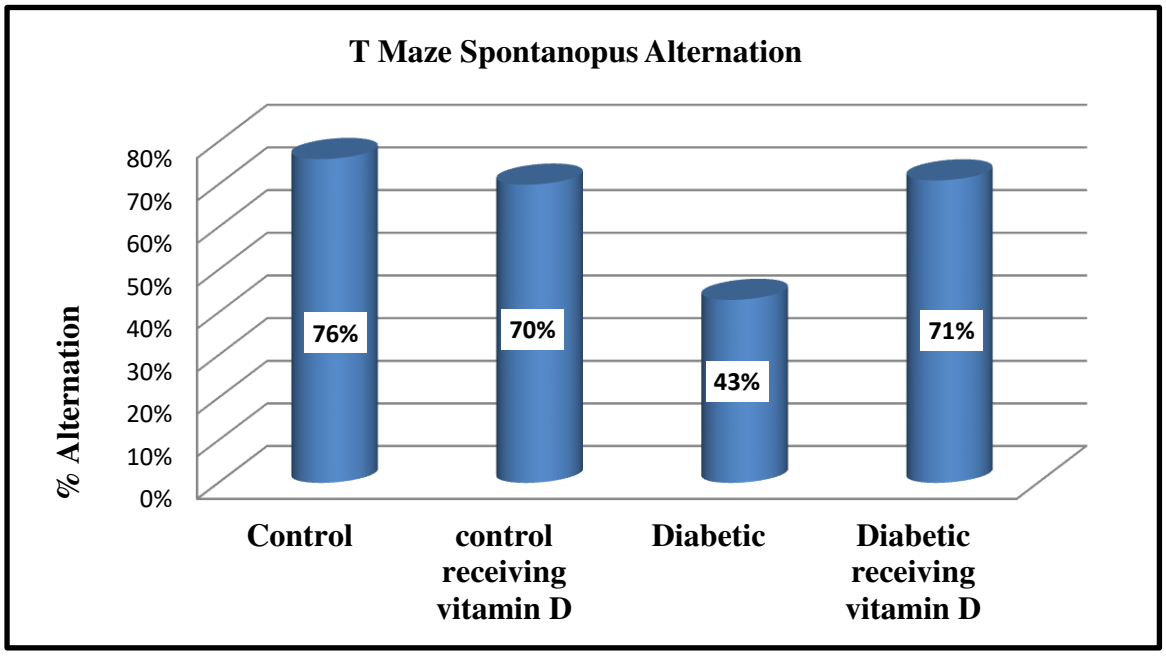

Fig. 1. The mean value of perncetage of alternation score in control, control receiving vitamin D, diabetic and diabetic receiving vitamin D.

B) Histological findings

H&E staining revealed normal structure of the hippocampal CA3 area in normal control and control receiving VD group, while the diabetic group showed abnormal morphologies of shrunken neurons with darkened, hyperchromatic, apoptotic nuclear changes, and even missing neurons. Surprisingly, vitamin D supplementation attenuated such neuronal damage **(Fig.2)**. These findings suggest that vitamin D supplementation attenuated T2 DM-induced neuronal death in the CA3 region of the hippocampus.

C) Expression of synaptophysin was investigated by immunohistochemistry

The expression of Synaptophysin in the CA3 region of the hippocampus in the normal control group and control receiving vitamin D showed with lowest reactivity to Synaptophysin antibodies than other groups **(Fig. 3 A and B),** however, diabetic group emphasized the highest expression of Synaptophysin along neurons, neuroglia cells, as well as neuropil **(Fig. 3 C)**. Surprisingly, the CA3 hippocampus in diabetic receiving vitamin D group displayed few Synaptophysin expressions compared to diabetic group **(Fig. 3 D)**.

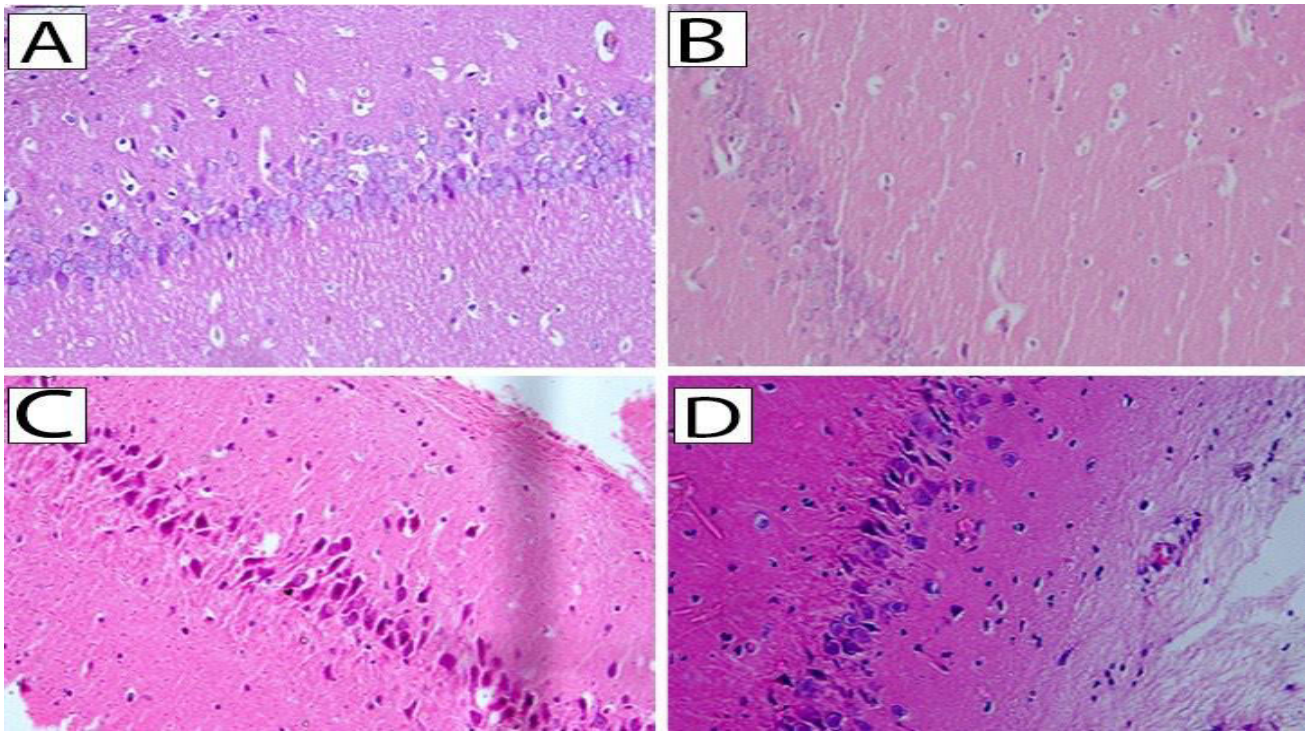

Fig.2. H&E staining of a rat brain from each group shows histological changes of the neural cells in a hippocampal CA3 region (x200). The normal control **(A)** and control receiving VD **(B)**, showed few layers of large pyramidal cells, most with vesicular nuclei; the hippocampal neurons were pyramidal, had a distinct, complete structure, and were well organized. The hippocampal neurons of diabetic group **(C)** showed disorganization with shrinkage dark nuclei of large pyramidal cells and areas of cell loss. the hippocampus of diabetic receiving VD rats **(D)** showed a protective effect in the form of preservation of large pyramidal cells and a decrease in the number of degenerated neurons.

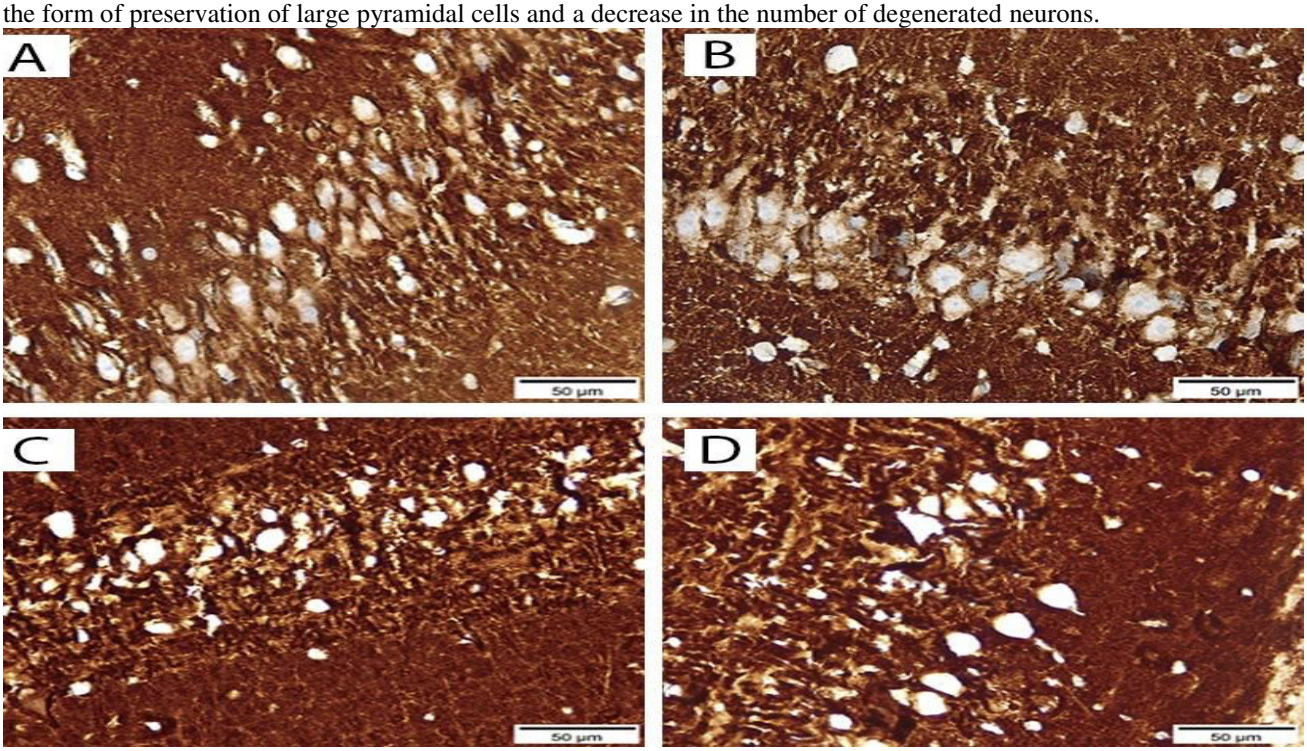

Fig.3. Photomicrographs highlighted the expression of Synaptophysin on the CA3 region of the hippocampus on brain tissues (x400) amongst assessed groups as follows: Section of CA3 hippocampus in normal control group **(A)** and control receiving vitamin D **(B)** emerged with lowest reactivity to Synaptophysin than other groups. Section of CA3 hippocampus of diabetic group **(C)** underscored the highest expression of Synaptophysin along neurons and neuroglia cells. Section of CA3 hippocampus of diabetic receiving VD **(D)** exhibited less reactivity to Synaptophysin compared to diabetic group.

D) Immunoscoring

In IHC staining, analysis of Synaptophysin optical density with significant differences among groups ($p = 0.001$) was demonstrated by one-way ANOVA. The mean value of optical density of Synaptophysin immunoreactivity in the hippocampus CA3 of diabetic group was dramatically elevated ($p < 0.001$; **Fig. 4**), compared with the control group. Compared with the diabetic group, vitamin D supplementation reduced Synaptophysin expression in the hippocampus CA3 ($p < 0.001$; **Fig. 4**).

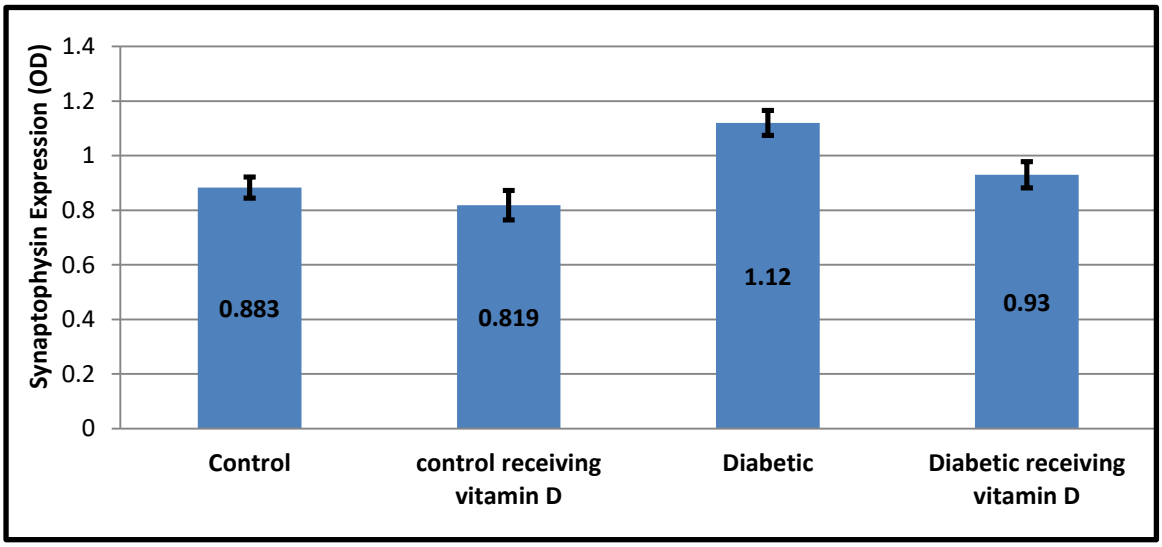

Fig.4. Mean value of optical density (OD) of Synaptophysin expression in the hippocampal tissue in control, control receiving VD, diabetic, and diabetic receiving VD.

Discussion

In the present study, we examined the effects of VD supplementation on improved cognitive deficits of high-fat diet (HFD) and STZ-induced T2DM model rats. Vitamin D-induced improvement in these effects was associated with decreasing hippocampal damage and expression of Synaptophysin in the hippocampus of T2DM model rat.

The hippocampus is a corner part of the limbic system, it is crucial for memory formation and emotional, reproductive, and adaptive behaviors **(Squire, 1992)**. Earlier findings of diabetic rats' hippocampus indicated that HFD diet-induced T2D triggered hippocampal neuronal death and revealed the presence of cell loss of a large pyramidal layer, All the layers are disorganized, and there are a lot of apoptotic cells **(Jaiswal et al., 2018)**.

The major findings in the current study are that diabetes induces apoptosis of CA3 pyramidal neurons in hippocampus of diabetic rats and revealed that, VD supplementation significantly enhanced hippocampal neuron survival in the HFD and STZ-induced-T2D rats.

In accordance with our results, a previous study showed that VD supplementation had neuroprotective effects in rat models of metabolic syndrome, which mediated through elevation of mGlut2 receptors and correction of various hippocampus areas' structural changes **(Alrefaie et al., 2022).** Another studies demonstrated that supplementation with the active form of vitamin D3-1,25-dihydroxy vitamin D3 improves metabolism, attenuates the pathological changes in hippocampus of diabetic rats and relieved increased number of the survived neurons and suppresses of apoptosis, which is mediated through relieving over-activation of endoplasmic reticulum (ER)

stress **(Yao et al., 2015; Haas et al., 2016; Guo et al., 2017)**.

To our knowledge, this research is the initial one to investigate the effect of VD on Synaptophysin expression in the hippocampus in a T2D rat model. We found that the Synaptophysin protein content in the hippocampal CA3 region from diabetic rats, as determined by immunohistochemistry is significantly increased when compared to control.

Synaptophysin is an integral membrane component of synaptic vesicles **(Arthur and Stowell , 2007)**. Synaptophysin was reported to be one of the inflammatory cytokines **(Su et al., 2018)**. **Gaspar et al. (2010)**, reported that Synaptophysin content increased in hippocampal nerve terminals of diabetic animals, as determined by Western blot analysis. These authors also suggested that the increase in the presynaptic Synaptophysin content might result from a compensatory mechanism of increased synaptic density that accompanies dendritic shortening caused by diabetes.

Another study reported that diabetes induces an increase in Synaptophysin in the granular layer of the cerebellum **(Sherif, 2017)** and in Rats' hippocampus suggests that synaptogenesis is currently occurring in these areas, or that synaptic protein in the existing synapses has been redistributed. Cognitive deficits were present along with these alterations, indicating that these synaptic reorganizations are aberrant **(Grillo et al., 2005)**. In cortical neurons exposed to high glucose, a recent study found that protein level of the presynaptic marker, Synaptophysin, rose. **(Sasaki-Hamada et al., 2022)**.

The corpus callosum and other white matter areas in the cuprizone-induced demyelination model showed lower levels of small physiological Synaptophysin dots and bigger spheroids, which can be termed pathological accumulations of synaptic vesicles in damaged axons. These spheroid structures are immunopositive for both Synaptophysin and amyloid precursor protein-hit matter structures, according to colocalization studies **(Gudi et al., 2017)**.

Increased synaptophysin immunoreactivity indicates accumulation of synaptic vesicles in presynaptic terminals at neuronal cell bodies and dendrites as well as associated with degeneration of neuronal cell bodies, their processes, and terminals. The histological basis for brain dysfunctions was provided by these findings, which showed suppression of synaptic vesicle transportation and synaptic dysfunction **(Shojo and Kibayashi, 2006)**..

Contrary to our findings, it was reported the hippocampus of diabetic rats displayed a reduced density of synaptic proteins syntaxin and Synaptophysin **(Duarte et al., 2009)**. The levels of Synaptophysin were decreased in hippocampal homogenates from hyperglycemic and recurrent hypoglycemic rats(**Cardoso et al., 2013**). However, this can be explained with the use of brain tissue homogenates for analysis, which are composed of different cell types such as neurons, endothelial cells, and astrocytes, which may mask the changes occurring at the neuronal level.

Conclusion

Our study demonstrates that VD supplementation ameliorates the pathological alterations in the hippocampus of diabetic rats, which provides evidence that VD that vitamin D supplementation can be a new protective option against neurodegenerative damage in patients with type 2 diabetes mellitus.

Recommendation

No more research was done on other brain structures due to time and financial constraints; nevertheless, in the future, we'll concentrate on looking at probable pathological mechanisms in other brain locations.

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