Vitamin D3 Improves Decline in Cognitive Function and Cholinergic Transmission in Prefrontal Cortex of Streptozotocin-Induced Diabetic Rats

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Abstract

Complications of diabetes mellitus include cognitive impairments and functional changes in the brain. Vitamin D has shown potential protective effects against many non-skeletal disorders. The present study aimed to investigate the effect of diabetes mellitus on episodic memory and cholinergic transmission in the prefrontal cortex. In addition, the possible beneficial effects of vitamin D3 were also assessed.

Methods: 30 male Wistar rats (150-200gm) were included into control, diabetic and diabetic supplemented with vitamin D3 groups. Diabetes was induced by single intraperitoneal injection of streptozotocin (stz) 45mg/kg in citrate buffer. Vitamin D3 was administered orally in a dose of 500 IU/kg/day in corn oil. 10 weeks after the onset of the treatment, rats were subjected to novel object recognition test to examine for episodic memory. Animals were sacrificed under diethyl ether anesthesia and prefrontal cortices were dissected to measure the activity of choline acetyl transferase (CAT) and acetycholine esterase (ACE) enzymes to assess for cholinergic transmission.

Results: Diabetic rats spent significantly less time exploring the novel object in novel object recognition test compared to control animals. Vitamin D3 significantly attenuated the diabetes-induced impairment such that animals again spent significantly more time exploring the novel object. The CAT activity was significantly decreased in diabetic animals while the ACE activity was significantly increased compared to control non-diabetic animals. Diabetes-induced alterations in enzyme activity in the prefrontal cortex were mitigated by vitamin D3 supplementation.

Conclusion: The present findings demonstrate that vitamin D3 improved the decline in cognitive function observed in diabetic animals. It is possible that this effect was mediated through enhancing the prefrontal cortex cholinergic transmission. The present work supports the importance of vitamin D3 supplementation to alleviate cognitive impairment associated with diabetes mellitus.

Key Words: Vitamin D3 – Streptozotocin.

Introduction

BRAIN is among the non-skeletal tissues that have been linked to vitamin D as the expression of vitamin D receptors was shown to be widespread in both neurons and glial cells [1].

Moreover, many recent findings have associated vitamin D deficiency to cognitive impairment and dementia [2]. Also active form of vitamin D has shown many neuroprotective effects [3].

Complications of diabetes mellitus have included neurophysiological and structural changes in the brain [4], furthermore, diabetes mellitus showed increased risk of learning and memory deficits besides to neurodegenerative diseases [5].

Several studies have been done trying to elaborate the pathophysiological mechanisms by which diabetes mellitus contributes to brain dysfunction.

Microvascular alteration, amyloid beta accumulation, oxidative stress and disturbances in neurotransmitters balance are among the suggested mechanisms [6]. In addition, hippocampal cell culture obtained from diabetic rats revealed dramatic impairment in neuronal survival, which could result in functional impairment in learning and memory [7].

There are many neurotransmitters which are essential for integrating functions of learning and memory in the brain including acetylcholine [8]. Alteration in cholinergic transmission in hippocampus and cortex was found to be related to cognitive impairment caused by cerebral hypoperfusion [9] and amyloid beta accumulation [10], however its relation to memory dysfunction in diabetic models has not been elucidated.
As the hypoglycemic drugs failed to reverse neurobehavioural, locomotor and learning impairments in diabetic models [11], a lot of measures have been investigated for possible preventive or modifying effects on neuro-psychological symptoms complicating diabetes mellitus.

The need to develop safe effective solutions is greatly indicated.

Aim:

The present study was designed to examine for the possible beneficial effects of vitamin D3 supplementation on episodic memory as a measure of cognitive function in stz-induced diabetes, and to investigate for alterations in markers of cholinergic transmission in the prefrontal cortex as a possible underlying mechanism.

Material and Methods

Animals:

Thirty male Wistar rats weighing 150-200gm, aged nearly 4 months were included in the present study. This age was chosen so as even after completing the study, animals would still be about 6-7 months old to exclude any possible effects of aging on cognitive function or neurotransmitter balance. Rats were obtained and maintained at the animal house, Kasr Alini Faculty of Medicine, Cairo University from May – October 2013. Animals were kept at room temperature under 12h light/dark cycle. They were allowed free access to rat chaw and water.

Chemicals:

Streptozotocin was obtained from MB Biomedicals LLA, California, USA. While vitamin D3 was obtained from Sigma-Aldrich Co., St. Louis, USA.

Experimental design:

Twenty rats were made diabetic by single intraperitoneal injection of streptozotocin (45mg/kg body weight) in citrate buffer (pH 4.5) [12]. Diabetes was confirmed by blood glucose levels above 250 mg/dl 72 hours following injection. The remaining 10 rats received intraperitoneal equivalent volume of citrate buffer and accounted for the control group.

Diabetic animals were classified into; diabetic group, and diabetic group supplemented with vitamin D3; rats were administered orally vitamin D3 in a dose of 5 00IU/kg/day in corn oil for 10 weeks [13].

Rats were assessed for their cognitive function using novel object recognition test.

Novel object recognition test:

Novel object recognition is a highly validated test for recognition memory in rodent models of CNS disorders. This test is useful for assessing impaired cognitive ability and evaluating different measures for their effect on cognition.

Rats show an innate preference for novel over familiar objects. The choice to explore the novel object reflects the use of learning and recognition memory. Rats readily approach objects and investigate them by touching and sniffing the objects and trying to manipulate them with their forepaws [14]. This behavior can be easily quantified and utilized to study episodic-like memory in rodents.

The test does not require lengthy training and does not induce high levels of arousal and stress like the water maze, it is more closely related to conditions under which human recognition memory is measured [15].

If memory is functioning normally, the rat will spend more time exploring the novel object than
it does exploring the familiar object. If exploration of both objects is the same, this can be interpreted as a memory deficit.

Previous studies designed forgetting curves which showed that control animals displayed good memory performance at 10 minutes to 2 hours inter-trial interval [16].

In the present experiment, the objects to be discriminated were made of plastic and were fixed to the floor to avoid being displaced by the rats. Each rat was exposed to a cage with two identical objects for 3 minutes then returned to its home cage. After 15 minutes inter-trial interval, the rat explored the cage in the presence of one familiar object and a novel object for 3 minutes. The time spent exploring each object was recorded. Exploration was defined as directly attending the object with the head while licking, sniffing or touching the object with its nose [17].

The total exploration time of both objects in both trials was calculated in addition to the discrimination index (DI).

\[
DI = \frac{\text{Exploration of novel object (EN)} - \text{Exploration of familiar object (EF)}}{\text{EN} + \text{EF}},
\]

the discrimination index represents the difference in exploration time expressed as a proportion of the total time spent exploring the two objects in the test trial [17].

Cholinergic transmission assessment:

Prefrontal cortical tissue was homogenized with ice-cold saline and centrifuged at 6000 RPM for 10min to obtain the supernatant that is to be used in the assessment.

Choline acetyl transferase (CAT): Measurement of CAT activity was performed using spectrophotometric method according to Chao [18]. 10ul of 0.5M sodium phosphate, 6mM acetyl coenzyme A, 1M choline chloride, 0.78mM methyl neostigmine sulphate, 3M sodium chloride, 1.1 mM EDTA, 20ul of 1M creatine HCL and 120ul distilled water were mixed together. The mixture was incubated at 37°C for 5min. 0.1ml of the supernatant obtained from prefrontal cortex homogenization was added to the mixture and kept at 37°C for 20min. Mixture was boiled for 5min to stop the reaction then added to 0.4ml distilled water. After cooling, the denatured protein was removed by centrifugation at 15000 RPM for 10min then 1 ul of 3mM dithiopyridine was added to 0.5ml of the new supernatant. The absorbance was read at 320nm and the level of enzyme activity was calculated from standard curve.

Acetyl choline esterase activity (ACE): The ACE activity was determined using colorimetric method according to Ellman et al., [19]. A mixture was prepared by adding 0.4ml aliquot of the supernatant obtained from prefrontal cortex homogenization and 2.6ml of phosphate buffer (0.1 M, pH8.0) and 100ul of dithiobisnitrobenzioc acid (DTNB). This mixture was incubated for 2min at 30°C, then the reaction was started with addition of acetylthiocholine (ATC) (30mM). ACE hydrolyzes ATC to thiocholine. The product of thiocholine reaction with DTNB was determined at 412nm for a period of 10min at 2min intervals for the absorbance per min.

Statistical analysis:

Data were statistically evaluated with SPSS for windows package version 20 (SPSS Inc., Chicago, IL, USA). Results are expressed as the Mean ±SD. One way analysis of variance (ANOVA) test was used to compare between groups. Pearson's correlation coefficient was carried out to assess association between discrimination index and activity of both CAT and ACE enzymes. Significance level at \(p < 0.05\) was considered to indicate statistical significance.

Results

Assessment of cognitive function was carried out by investigating performance in the novel object recognition test. Total exploration times for the two objects were noted during both training and test sessions. There were no significant differences in total exploration time between different groups.
Control rats spent more time exploring the novel object than the familiar object during the test session. In contrast, diabetic rats spent significantly less time exploring the novel object as the DI decreased from 0.43 ± 0.028 in the control group to 0.26 ± 0.033 (p < 0.001) in the diabetic group. This observation clearly indicates that diabetes resulted in memory deficits in the novel object recognition test.

Supplementation of diabetic animals with vitamin D3 mitigated the decline in memory function and increased the DI of diabetic animals to 0.31 ± 0.031 (p < 0.01) when compared to diabetic group.

Trying to investigate for the possible alterations underlying the deterioration in cognitive function, the present study revealed significant decrease in CAT enzyme activity in contrast to ACE activity which got significantly increased (p < 0.001 for both enzymes) in prefrontal cortices of diabetic rats compared to control rats indicating decreased production and increased degradation of acetylcholine with consequent reduction in the efficiency of cholinergic neurotransmission.

Treatment of diabetic rats with vitamin D3 significantly reversed the decrease in CAT and the increase in ACE activities (p < 0.01) indicating relative enhancement of cholinergic transmission in the prefrontal cortices of vitamin D3-treated diabetic rats.

Correlation studies performed between discrimination index (DI) and activity levels of CAT and ACE enzymes revealed negative association between DI and activity of ACE enzyme. Although this association didn't reach significance, but it clarifies that increased activity of ACE which leads to decreased availability of acetylcholine in the prefrontal cortex would be associated with lower DI and decreased preference to novel object recognition indicating poor memory function (Table 1, Figs. 1-4).

Table 1: Choline acetyl transferase activity CAT (u/mg), acetyl choline esterase activity ACE (nmol/min/g), total exploration time (sec) and discrimination index DI in all groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>CAT activity</th>
<th>ACE activity</th>
<th>Total exploration time</th>
<th>Discrimination index DI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>30.58±5.56</td>
<td>1.71±0.33</td>
<td>85.25±6.99</td>
<td>0.43±0.028</td>
</tr>
<tr>
<td>Diabetic</td>
<td>19.27±3.68</td>
<td>3.56±0.96</td>
<td>76.18±8.38</td>
<td>0.26±0.033</td>
</tr>
<tr>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetic+</td>
<td>26.01±4</td>
<td>2.40±0.51</td>
<td>76.7±9.11</td>
<td>0.31±0.031</td>
</tr>
<tr>
<td>vit D</td>
<td>p&lt;0.01</td>
<td>p&lt;0.01</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

p1: Significance when compared to control group.
p2: Significance when compared to diabetic group.
NS: Non significant.
Discrimination index (between novel and familiar objects in all groups)

<table>
<thead>
<tr>
<th>Group</th>
<th>Control group</th>
<th>Diabetic group</th>
<th>Diabetic group + vit D</th>
</tr>
</thead>
<tbody>
<tr>
<td>p1</td>
<td>Significant when compared to control group.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p2</td>
<td>Significant when compared to diabetic group.</td>
<td></td>
<td></td>
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Fig. (4): Discrimination index DI in all groups.

Discussion

Findings of the present work revealed significant decrease in preference for novel objects in stz-induced diabetic rats. Diabetic animals also showed significant depression in cholinergic transmission in their prefrontal cortices. Vitamin D3 supplementation to diabetic rats enhanced their memory function and improved alterations in the markers of cholinergic transmission. The aforementioned results suggest that vitamin D3 supplementation improved the diabetes-induced modulation in prefrontal cortex cholinergic transmission with resultant improvement in cognitive function.

The novel object recognition test depends on the ability to judge prior occurrence of an event. It utilizes the perceptual features of the object besides to self involvement in the experience. This test examines for episodic memory which is subtype of declarative or cognitive memory. It not only extends to the past in the form of remembering, but also involves thinking and planning. That is why testing for episodic memory is considered valid and efficient for assessing cognitive function.

In the present work, performance in the novel object recognition test was significantly impaired in diabetic rats as compared to control rats. It is to be noted that the total exploration time was insignificantly different between the study groups; this confirms that the poor performance in the test was unrelated to defect in motivation or sensory-motor deficits but caused by cognitive impairment.

The corresponding cognitive defects observed in diabetic patients and stz-induced diabetic rats provide evidence which support the present findings.

Type 2 diabetic patients performed significantly worse than control subjects in memory and information processing tests in the study of Tiehuis et al., [23].

In addition, the meta-analysis performed by Kálcza-Jánosi K et al., [24], which included 3studies on type 1 and 6 studies on type 2 diabetic patients; showed that patients of both types of diabetes suffered of reduced performance in numerous cognitive domains.

Regarding stz-induced diabetic rats; several studies observed potential changes in their cognitive functions and considered diabetes as a high risk factor to dementia [25,26].

Diabetes induced functional disorders in the brain were attributed to neurochemical, neurophysiological and structural abnormalities [27]. Further researches are needed to elaborate the mechanisms underlying cognitive deficits in diabetes mellitus.

The present findings suggest that diminished cholinergic transmission in the prefrontal cortex could be among possible underlying mechanisms.

In accord with the present hypothesis, Dere et al., [28], showed that modulation of cholinergic neurotransmission in rodents has an impact on the acquisition and possibly also the consolidation of object information leading to impairment of object recognition.

Also, Bhutada et al., [29], observed that treatment with ACE inhibitor improved the diabetes induced cognitive dysfunction in rat model of stz-induced diabetes.

Cholinergic projections originating in the basal forebrain innervate supragranular (layers I and II) and infragranular (layers V and VI) layers of the prefrontal cortex and acetylcholine release in the frontal cortex has been implicated in novelty-induced arousal, attention and the encoding of novel stimuli and memory consolidation [30].

Moreover, Wallacea and Bertrand [31], observed that depletion of acetyl choline in the prefrontal cortex was shown to produce profound impairments in cognitive functions, while the enhancement of cholinergic transmission have showed promnestic effects [32].

In addition, a great deal of evidence suggested that the depletion of neocortical acetylcholine
contributes to the memory deficits observed in Alzheimer’s disease [33].

Utilization of vitamin D3 supplementation in the prevention and treatment of many non-skeletal disorders has been much increased.

No previous work has investigated the effect of vitamin D3 on cognitive function in diabetic rats. Moreover the hypoglycemic drugs failed to reverse most of neurobehavioral disruptions observed in diabetes, that is why the need to find adjuvant treatment that can manage such neuro-dysfunctions is highly demanded.

In a longitudinal observational study done by Obermann et al., [34], in normal population to explore effects of commonly used drugs and supplements on cognition, vitamin D supplementation showed association with improved cognitive performance. Furthermore, Annweiler [35], observed association between episodic memory disorders and lower vitamin D levels in the meta-analysis performed on many studies in normal population. Also, the risk of cognitive impairment was up to 4 times greater in vitamin D-severely deficient individuals (<25nmol/L) as stated by Soni et al., [3].

These recent observations support the observation of the present work that vitamin D3 can protect against diabetes-induced cognitive dysfunction.

The present work extended to highlight the positive impact of vitamin D3 on cholinergic transmission in the prefrontal cortex.

Some previous works tried also to investigate for mechanisms of neuro-protective effects of vitamin D.

Vitamin D3 was shown to stimulate clearance of amyloid beta plaques which trigger cortical neurodegeneration, protect against apoptosis, exhibit antioxidant effects and up regulate production of several neurotrophic factors that promote neuronal survival and function [36,37].

In addition, vitamin D decreased the pro-inflammatory and increased the anti-inflammatory cytokines in brains of aged rats [38].

**Conclusion:**

The present study confirms cognitive deterioration in diabetes mellitus and postulates important mechanism that is the decline in cholinergic transmission in the prefrontal cortex. The present findings also give evidence that vitamin D3 can offer protection against decline in cognitive function and highlight the importance of vitamin D3 supplementation in diabetic patients.

**References**


