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Cholinergic, dopaminergic and insulin receptors gene expression in the cerebellum of streptozotocin-induced diabetic rats: Functional regulation with Vitamin D_3 supplementation

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ABSTRACT

The study was to find out the effect of Vitamin D_3 supplementation on preventing the altered gene expression of cholinergic, dopaminergic, insulin receptors and GLUT3 gene expression in cerebellum of diabetic rats. Radioreceptor binding assays and gene expression were done in the cerebellum of male Wistar rats. Rota rod has been used to evaluate motor coordination. Our results showed a significantly increased gene expression of dopamine D2, muscarinic M1, M3, α7 nicotinic acetylcholine, insulin receptors, acetylcholine esterase, GLUT3 and Vitamin D receptor in the cerebellum of diabetic rats. There was a down-regulation of dopamine D1 receptor. Total dopamine receptor showed a decreased and total muscarinic, muscarinic M1 and M3 receptors showed an increased binding parameter, B_{max}. Rota rod experiment showed a significant decrease in the retention time on the rotating rod in diabetic while treatment improved retention time near to control. Vitamin D_3 and insulin treatment markedly recovered the altered gene expression and binding parameters to near control. Our study showed Vitamin D_3 functional regulation through dopaminergic, cholinergic and insulin receptors and glucose transport mechanism through GLUT3 in the cerebellum of diabetic rats which play a major role in neuroprotection in diabetes which has clinical application.

1. Introduction

Vitamin D_3 is traditionally recognized as a potent regulator of calcium and phosphorus metabolism. Vitamin D_3 is either synthesised in the epidermis from 7-dehydrocholesterol by the absorption of ultraviolet light, or obtained from the diet in a limited number of foods such as eggs, fish oils, and fortified milk (DeLuca, 1993). Hyperglycaemia during uncontrolled diabetes is known to cause oxidative stress, which has been implicated in various secondary complications of diabetes. Diabetes mellitus has been reported to be accompanied by a number of behavioral and hormonal abnormalities, including hyperphagia, reduced motor activity (Marchall et al., 1976; Marchall, 1978). The biological actions of Vitamin D_3 are mediated through binding to the Vitamin D receptor (VDR), a member of the nuclear steroid hormone receptor family (Strugnell and DeLuca, 1997). An increased prevalence of diabetes has been described in Vitamin D-deficient individuals (Boucher et al., 1995; Isaia et al., 2001; Chiu et al., 2004). Insulin synthesis and secretion has been shown to be impaired in β cells in Vitamin D-deficient animals.

In the cerebellum, nicotinic acetylcholine receptors mediate the release of glutamate (Reno et al., 2004), GABA (De Filippi et al., 2001; Rossi et al., 2003) and norepinephrine (O'Leary and Leslie, 2003). These receptors significantly influence the activity within the cerebellar circuitry, and any deregulation of this activity contributes to functional disorders involving the cerebellum. Diabetes is also found to be associated with changes in somatic sensations which involve the cerebellum, cerebral cortex and thalamus. Symptoms, like loss of pain, impaired touch perception and decreased position sense, have been commonly documented in a diabetic patient (Waxman and Sabin, 1981). Atrophy of the cerebellum has been reported in diabetic patients, and this is not associated with the duration of the disease or glycaemic control (Lunetta et al., 1994).

Dopamine in the central nervous system is involved in the control of both motor and emotional behavior (Vallone et al., 2000) and peripherally modulates insulin secretion in the pancreatic islets (Nogueira et al., 1994). Nafadotride, a preferential antagonist of dopamine D3 receptors administered at low doses directly into the cerebellum, has been shown to activate locomotor activity (Barik and de Baurepaire, 1996).

Acetylcholine is a major neurotransmitter of the peripheral parasympathetic nervous system. It helps to facilitate the release of insulin in a glucose-dependent mode. Hence this activity has been

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shown to be mediated by the activation of muscarinic acetylcholine receptors located on the pancreatic β cells (Ahren, 2000; Gilon and Henquin, 2001). The brain glucose uptake is ultimately dependent on facilitative glucose transporters, the modulation of brain glucose transporters intrinsic activity. GLUT3 is the main neuronal glucose transporter (Maher et al., 1993) abundant in the brain.

Immunohistochemistry showed the presence of VDR in pituitary cells and mRNA and protein VDR expression in human pituitary gland (Perez-Fernandez et al., 1997), suggesting a possible role of Vitamin D in regulation of the brain endocrine system. A putative receptor for 1,25(OH)2D has been detected in chick brain (Jia and Nemere, 1999), allowing speculation that 1,25(OH)2D could act like other neuroactive hormones in modulating neuronal activity and neurotransmitter receptors (Rupprecht and Holsboer, 1999; Zakon, 1998). It is of particular importance that VDR and catalytic enzymes are colocalized in the brain (Baulieu, 1998), supporting an autocrine/paracrine function for Vitamin D. These findings support a functional role for Vitamin D in the human brain (McGrath et al., 2001).

The role of Vitamin D_3 in regulating the cholinergic and dopaminergic receptors function in the cerebellum has not been studied. In the present study we examine the effect of Vitamin D_3 in modulating the cholinergic, dopaminergic and insulin receptors and GLUT3 in the cerebellum of STZ-induced diabetic rats for understanding the therapeutic role of Vitamin D_3 in diabetes associated functional disorders involving the cerebellum. Our present study on the anti-diabetic property of Vitamin D_3 in cerebellum mediated through cholinergic and dopaminergic receptors will definitely enlighten novel therapeutic possibilities for diabetes.

2. Materials and methods

Bio chemicals used in the present study were purchased from Sigma Chemical Co., St. Louis, USA. All other reagents of analytical grade were purchased locally. Quinuclidinyl benzilate, L-[Benzilic-4,4′-3H], ([^{3}H] QNB)(Sp. Activity 42 Ci/mmol), 4-DAMP, [N-methyl-3H] (Sp. Activity 83 Ci/mmol), [^{3}H] dopamine were purchased from NEN Life Sciences Products Inc., Boston, U.S.A. Pirenzepine, 4-DAMP mustard, dopamine and cholecalciferol were from Sigma Chemical Co., USA. Tri-reagent kit was purchased from MRC, USA. Real Time PCR Taqman probe assays on demand were from Applied Biosystems, Foster City, CA, USA.

Male adult Wistar rats of 180–240 g body weight were used for all experiments. The animals were allowed to acclimatise for 2 weeks before the experiment. They were housed individually in separate cages under 12 h light and 12 h dark periods. Rats had free access to standard food and water ad libitum. All animal care and procedures were done in accordance with the Institutional and National Institute of Health guidelines. All efforts were made to minimize the number of animals used and their suffering. Diabetes was induced in rats by single intra femoral vein injection of STZ freshly dissolved in 0.1 M citrate buffer, pH 4.5, under anaesthesia (Junod et al., 1969). STZ was given at a dose of 55 mg/kg body weight (Hohenegger and Rudas, 1971; Arison et al., 1967). Animals were divided into the following groups: (i) Control, (ii) diabetic, (iii) insulin-treated diabetic and (iv) Vitamin D_3-treated diabetic rats. Each group consisted of 6–8 animals. The insulin-treated diabetic group received subcutaneous injections (1 U/kg body weight) of Lente and Plain insulin (Boots India) daily during the entire period of the experiment. The last injection was given 24 h before sacrificing the rats. Vitamin D_3-treated groups received 12 μg/kg Vitamin D_3 dissolved in 0.3 ml of coconut oil. The supplementation was administrated *via* gavage for a period of 2 weeks (De Souza Santos and Marques Vianna, 2005) for the entire period of the experiment. Rats were sacrificed on 15th day by decapitation. The cerebellum was dissected out quickly over ice according to the procedure of Glowinski and Iversen (1966), and the tissues collected were stored at −80 °C until assayed.

2.1. Estimation of blood glucose

Blood glucose was estimated by the spectrophotometer method using glucose oxidase–peroxidase reactions. Blood samples were collected from the tail vein at 0 h (Before the start of the experiment), 3rd, 6th, 10th and 14th day and the glucose levels were estimated subsequently. Along with this blood samples were collected 3 h after the administration of morning dose of insulin and Vitamin D_3. The results were expressed in terms of milligram per decilitre of blood.

2.2. Rota rod test

Rota rod has been used to evaluate motor coordination by testing the ability of rats to remain on revolving rod (Dunham and Miya, 1957). The apparatus has a horizontal rough metal rod of 3 cm diameter attached to a motor with variable speed. This 70 cm long rod was divided into four sections by wooden partitions. The rod was placed at a height of 50 cm to discourage the animals to jump from the rotating rod. The rate of rotation was adjusted in such a manner that it allowed the normal rats to stay on it for 5 min. Each rat was given five trials before the actual reading was taken. The readings were taken at 10, 15 and 25 rpm after 15 days of treatment in all groups of rats.

2.2.1. Total muscarinic, muscarinic M1 and M3 receptor binding studies in the cerebellum

Binding assay in cerebellum was done according to the modified procedure of Yamamura and Synder (1981), Cerebellum was homogenised in a polytron homogeniser with 20 volumes of cold 50 mM Tris–HCl buffer, pH 7.4 containing 1 mM EDTA. The supernatant was then centrifuged at 30,000×*g* for 30 min and the pellets were resuspended in appropriate volume of Tris–HCl-EDTA buffer pH 7.4.

Total muscarinic, and muscarinic M1 receptor binding parameter assays were done using [^{3}H]QNB (0.1–2.5 nM) and M3 receptor using [^{3}H]DAMP (0.01–5 nM). The non-specific binding was determined using 100 μM atropine for Total muscarinic, pirenzepine for muscarinic M1 and 4-DAMP for M3 receptor. Total incubation volume of 250 μl contains 200–250 μg protein concentrations. Tubes were incubated at 22 °C for 60 min and filtered rapidly through GF/C filters (Whatman). The filters were washed quickly by three successive washing with 5.0 ml of ice cold 50 mM Tris–HCl buffer, pH 7.4. Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter. The non-specific binding determined showed 10% in all our experiments.

2.2.2. Total dopamine receptor binding studies in the cerebellum

DA receptor assay was done using [^{3}H]DA according to Madras et al. (1988). Cerebellum was homogenised in a polytron homogeniser with 20 volumes of cold 50 mM Tris–HCl buffer, along with 1 mM EDTA, 0.01%ascorbic acid, 4 mM $MgCl_2$, 1.5 mM $CaCl_2$, pH 7.4 and centrifuged at 38,000×*g* for 30 min at 4 °C. The pellet was washed twice by rehomogenization and centrifuged twice at 38,000×*g* for 30 min at 4 °C. This was resuspended in appropriate volume of the buffer containing the above mentioned composition.

Binding assays were done using different concentrations i.e., 0.25 nM–1.5 nM of [^{3}H]DA in 50 mM Tris–HCl buffer, along with 1 mM EDTA, 0.01% ascorbic acid, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 120 mM NaCl, 5 mM KCl, pH 7.4 in a total incubation volume of 250 μl containing 200–300 μg of proteins. Specific binding was determined using 100 μM unlabelled dopamine.

Tubes were incubated at 25 °C for 60 min. and filtered rapidly through GF/B filters (Whatman). The filters were washed quickly by three successive washing with 5.0 ml of ice cold 50 mM Tris buffer, pH 7.4. Bound radioactivity was counted with cocktail-T in a Wallac 1409 liquid scintillation counter. The non-specific binding determined showed 10% in all our experiments.

2.3. Protein determination

The amount of protein was measured by the method of Lowry et al. (1951) using bovine serum albumin as standard. The intensity of the purple blue colour formed was proportional to the amount of protein, which was read in a spectrophotometer at 660 nm.

2.4. Receptor data analysis

The receptor binding parameters were determined using Scatchard analysis (Scatchard, 1949). The specific binding was determined by subtracting non-specific binding from the total. The binding parameters, maximal binding (B_{max}) and equilibrium dissociation constant (K_d), were derived by linear regression analysis by plotting the specific binding of the radioligand on X-axis and bound/free on Y-axis using Sigma plot software (version 2.0, Jandel GmbH, Erkrath, Germany). The maximal binding is a measure of the total number of receptors present in the tissue and the equilibrium dissociation constant is the measure of the affinity of the receptors for the radioligand. The K_d is inversely related to receptor affinity.

2.4.1. Analysis of gene expression by real time PCR

RNA was isolated from the cerebellum of experimental rats using the Tri-reagent (MRC, USA). Total cDNA synthesis was performed using ABI PRISM cDNA archive kit in 0.2 ml microfuge tubes. The reaction mixture of 20 µl contained 0.2 µg total RNA, 10× RT buffer, 25× dNTP mixture, 10× random primers, MultiScribe RT (50 U/µl) and RNase free water. The cDNA synthesis reactions were carried out at 25 °C for 10 min and 37 °C for 2 h using an Eppendorf Personal Cycler. Real-time PCR assays were performed in 96-well plates in ABI 7300 real-time PCR instrument (Applied Biosystems). The primers and probes were purchased from Applied Biosystems, Foster City, CA, USA. The TaqMan reaction mixture of 20 µl contained 25 ng of total RNA-derived cDNAs, 200 nM each of the forward primer, reverse primer and TaqMan probe for Muscarinic M1 receptor gene and endogenous control β-actin and 12.5 µl of Taqman 2X Universal PCR Master Mix (Applied Biosystems) and the volume was made up with RNAse free water. The following thermal cycling profile was used (40 cycles): 50 °C for 2 min, 95 °C for 10 min, 95 °C for 15 s and 60 °C for 1 min.

Fluorescence signals measured during amplification were considered positive if the fluorescence intensity was 20-fold greater than the standard deviation of the baseline fluorescence. The $^{\Delta\Delta}$CT method of relative quantification was used to determine the fold change in expression. This was done by normalizing the resulting threshold cycle (CT) values of the target mRNAs to the CT values of the internal control β-actin in the same samples ($^{\Delta}CT = CT_{Target} - CT_{\beta\text{-actin}}$). It was further normalized with the control ($^{\Delta\Delta}CT = {}^{\Delta}CT - CT_{Control}$). The fold change in expression was then obtained as ($2^{-\Delta\Delta}CT$) and the graph was plotted using log $2^{-\Delta\Delta}CT$.

2.5. Statistics

Statistical evaluations were done by ANOVA, expressed as mean ± S.E.M using In Stat (Ver.2.04a) computer programme.

Table 2
Rota rod performance of control, diabetic, D + I and D + V rats.

Animal status	Retention time on the rod (in seconds)		
	10 rpm	15 rpm	25 rpm
Control	116.00 ± 4.82	111.43 ± 3.30	65.54 ± 4.23
Diabetic	83.33 ± 3.20$^{@@@}$	55.23 ± 3.35$^{@@@}$	33.63 ± 3.75$^{@@@}$
D + I	106.00 ± 4.70***	114.45 ± 3.70***	70.00 ± 7.42***
D + V	102.00 ± 6.12***	85.52 ± 4.48**	62.23 ± 4.95**

Values are mean ± S.E.M of 4–6 separate experiments (n = 5–6 rats per group) ANOVA followed by Students–Newman–Keuls' test. $^{***}p<0.001$, $^{**}p<0.01$ $^{@@@}p<0.001$, and $^{**}p<0.01$ when compared to diabetic rats.
D + I — Insulin-treated diabetic.
D + V — Vitamin D_3-treated diabetic.

3. Results

Blood glucose level of all rats before STZ administration was within the normal range. STZ administration led to a significant increase ($p<0.001$) in blood glucose level of diabetic rats compared to control rats. Insulin and Vitamin D_3 treatment were able to significantly reduce ($p<0.001$) the increased blood glucose level to near the control value compared to diabetic group (Table 1).

3.1. Rota rod performance of control and experimental groups of rats

Rota rod experiment at 10, 15 and 25 revolutions per minute (rpm) showed a significant decrease ($p<0.01$) in the retention time on the rotating rod in the diabetic group compared to control. Both insulin treatment and Vitamin D_3 treatment to diabetic rats significantly reversed the retention time near to control at 10 ($p<0.01$), 15 ($p<0.01$) and 25 ($p<0.05$) rpm (Table 2).

3.2. Total muscarinic receptor analysis

3.2.1. Scatchard analysis of [^{3}H] QNB binding against atropine in the cerebellum of control and experimental rats

The Scatchard analysis showed that the B_{max} and K_d of the [^{3}H]QNB receptor binding increased significantly ($p<0.001$) in the cerebellum of diabetic rats compared to control group. In Vitamin D_3 and insulin-treated diabetic groups, B_{max} reversed to near control value. K_d of insulin-treated group reversed to near control and Vitamin D_3 treatment shows no significance in K_d (Table 3).

3.3. Muscarinic M1 receptor analysis

3.3.1. Scatchard analysis of [^{3}H]QNB binding against pirenzepine in the cerebellum of control and experimental rats

The Scatchard analysis showed that the B_{max} and K_d of muscarinic M1 receptors of Cerebellum were increased significantly ($p<0.001$) in diabetic condition compared to control group. Insulin and Vitamin D_3-treated diabetic rats B_{max} and K_d were reversed to near control value compared to diabetic group (Table 3).

Table 1
Blood glucose (mg/dl) level in control, diabetic, D + I and D + V rats.

Animal status	0 day (before STZ injection)	3rd day (initial)	6th day	10th day	14th day (final)
Control	86.2 ± 1.4	93.5 ± 1.6	89.4 ± 0.8	101.2 ± 2.2	97.7 ± 1.21
Diabetic	79.4 ± 1.5	253.1 ± 0.5	315.1 ± 1.2	309.7 ± 0.6	311.9 ± 1.4***
D + I	85.2 ± 0.8	256.8 ± 0.5	303.6 ± 0.7	190.9 ± 1.5	137.0 ± 1.3$^{\Psi\Psi\Psi}$ $^{\Phi\Phi\Phi}$
D + V	84.2 ± 1.2	84.2 ± 1.2	310 ± 0.8	213 ± 1.5	190.2 ± 1.4$^{\Psi\Psi\Psi}$ $^{\Phi\Phi\Phi}$

Values are mean ± S.E.M of 4–6 rats in each group. Each group consist of 6–8 rats. $^{***}p<0.001$ when compared to control, $^{\Psi\Psi\Psi}p<0.001$ when compared to diabetic group, and $^{\Phi\Phi\Phi}p<0.001$ when compared to initial reading.
D + I — Insulin-treated diabetic.
D + V — Vitamin D_3-treated diabetic.

Table 3
Binding parameters of total muscarinic, muscarinic M1, M3 and total dopamine receptor in the cerebellum of control, diabetic, D + I and D + V rats.

Animal status	B_{max} (fmoles/mg protein)				K_d (nM)			
	Total muscarinic	Muscarinic M1	Muscarinic M3	Total dopamine	Total muscarinic	Muscarinic M1	Muscarinic M3	Total dopamine
Control	65 ± 6.1	192 ± 12.4	11 ± 1.4	112 ± 5.4	0.60 ± 0.02	0.55 ± 0.02	0.25 ± 0.02	3.8 ± 0.14
Diabetic	160 ± 9.2***	294 ± 13.2***	43 ± 2.2***	22 ± 3.6***	1.1 ± 0.02**	0.98 ± 0.02***	1.05 ± 0.02***	2.3 ± 0.05**
D + I	84 ± 5.5ψψψ	210 ± 8.6ψψψ	9 ± 0.5ψψψ	116 ± 4.3ψψψ	0.65 ± 0.01@@	0.50 ± 0.01@@	0.3 ± 0.01@@	3.2 ± 0.13@@
D + V	115 ± 7.6ψψ	210 ± 8.4ψψψ	14 ± 0.7ψψψ	114 ± 6.5ψψψ	1.06 ± 0.04	0.47 ± 0.03@@	0.33 ± 0.03@@	2.9 ± 0.09@@

Values are mean ± S.E.M of 4–6 separate experiments. Each group consist of 6–8 rats. ***$p<0.001$ when compared to control, ψψψ$p<0.001$ when compared to diabetic group, **$p<0.01$ when compared to control, ψψ$p<0.01$ when compared to diabetic group, and @@$p<0.01$ when compared to diabetic group.
D + I — Insulin-treated diabetic.
D + V — Vitamin D_3-treated diabetic.

3.4. Muscarinic M3 receptor analysis

3.4.1. Scatchard analysis of [^{3}H] DAMP binding against 4-DAMP mustard in the cerebellum of control and experimental rats

The Scatchard analysis showed that the B_{max} and K_d of muscarinic M3 receptors of cerebellum were increased significantly ($p<0.001$) in diabetic rats compared to control group. Insulin and Vitamin D_3-treated diabetic rats showed B_{max} and K_d were reversed to near control value compared to diabetic group (Table 3).

3.5. Total dopamine receptor analysis

3.5.1. Scatchard analysis of [^{3}H] dopamine binding against dopamine in the cerebellum of control and experimental rats

The Scatchard analysis showed that the B_{max} and K_d of the [^{3}H] dopamine receptor binding decreased significantly ($p<0.001$) in the cerebellum of diabetic rats compared to control group. In Vitamin D_3 and insulin-treated diabetic groups, B_{max} reversed to near control value. K_d of insulin-treated group reversed to near control and Vitamin D_3 treatment shows no significance in K_d (Table 3).

3.6. Real time-PCR analysis of muscarinic M1 receptor

Real Time-PCR analysis showed that the muscarinic M1 receptor gene expression in the cerebellum was increased significantly ($p<0.001$) in diabetic condition and it reversed to near control value in insulin and Vitamin D_3-treated diabetic rats (Table 4).

3.7. Real time-PCR analysis of muscarinic M3 receptor

Real Time-PCR analysis showed that the muscarinic M3 receptor gene expression in the cerebellum was increased significantly ($p<0.001$) in diabetic condition and it reversed to near control value in insulin and Vitamin D_3-treated diabetic rats (Table 4).

3.8. Real time-PCR analysis of α7 nicotinic acetylcholine receptor

Real Time-PCR analysis showed that α 7 nicotinic acetylcholine receptor gene expression in the cerebellum was increased significantly ($p<0.001$) in diabetic condition and it reversed to near control value in Vitamin D_3-treated diabetic rats. But insulin treatment did not show any significant change in α7 nicotinic acetylcholine receptor gene expression in the cerebellum when compared to diabetes (Table 4).

3.9. Real time-PCR analysis of acetylcholine esterase

Real Time-PCR analysis showed that the acetylcholine esterase gene expression in the cerebellum was increased significantly ($p<0.001$) in diabetic condition and it reversed to near control value in insulin and Vitamin D_3-treated diabetic rats (Table 4).

3.10. Real time-PCR analysis of dopamine D1 receptor

Real Time-PCR analysis showed that the dopamine D1 receptor gene expression in the cerebellum was decreased significantly ($p<0.001$) in diabetic condition and it reversed to near control value in insulin and Vitamin D_3-treated diabetic rats (Table 4).

3.11. Real time-PCR analysis of dopamine D2 receptor

Real Time-PCR analysis showed that the dopamine D2 receptor gene expression in the cerebellum was increased significantly ($p<0.001$) in diabetic condition and it reversed to near control value in insulin and Vitamin D_3-treated diabetic rats (Table 4).

3.12. Real time-PCR analysis of insulin receptor

Real Time-PCR analysis showed that the insulin receptor gene expression in the cerebellum was increased significantly ($p<0.01$) in

Table 4
Real time amplification of mRNA from the cerebellum of control, diabetic, D + I and D + V rats.

Experimental groups	Log RQ value								
	Muscarinic M1 receptor	Muscarinic M3 receptor	α7 nicotinic receptor	Acetylcholine esterase	Dopamine D1 receptor	Dopamine D2 receptor	Insulin receptor	GLUT3	VDR
Control	0	0	0	0	0	0	0	0	0
Diabetic	6.97 ± 1.56[a]	7.48 ± 1.22[a]	0.25 ± 0.04[a]	3.03 ± 0.32[a]	−0.33 ± 0.04[a]	0.87 ± 0.32[a]	0.42 ± 0.32[a]	0.37 ± 0.07[a]	0.62 ± 0.05[a]
Insulin-treated diabetic group	0.91 ± 0.72[b]	1.33 ± 0.22[b]	0.22 ± 0.03	0.57 ± 0.04[b]	0.08 ± 0.03[b]	−0.4 ± 0.03[b]	−0.02 ± 0.02[b]	0.03 ± 0.03[b]	−0.2 ± 0.03[b]
Vitamin D_3-treated diabetic group	0.65 ± 0.065[b]	0.8 ± 0.5[b]	−0.02 ± 0.04[b]	−0.37 ± 0.04[b]	0.06 ± 0.02 [b]	−0.17 ± 0.05[b]	0.29 ± 0.1[b]	−0.05 ± 0.04[b]	−0.12 ± 0.03[b]

Values are mean ± S.E.M of 4–6 separate experiments. Each group consist of 6–8 rats. Relative quantification values and standard deviations are shown in the table. The relative ratios of mRNA levels were calculated using the $^{\Delta\Delta}$CT method normalized with β-actin CT value as the internal control and control CT value as the calibrator. [a]$p<0.001$ when compared with control and [b]$p<0.001$ when compared with diabetic group.
D + I — Insulin-treated diabetic.
D + V — Vitamin D_3-treated diabetic.

diabetic condition and it reversed to near control value in insulin and Vitamin D_3-treated diabetic rats (Table 4).

3.13. Real time-PCR analysis of GLUT3 receptor

Real Time-PCR analysis showed that the GLUT3 gene expression in the cerebellum was increased significantly ($p<0.001$) in diabetic condition and it reversed to near control value in insulin and Vitamin D_3-treated diabetic rats (Table 4).

3.14. Real time-PCR analysis of VDR receptor

Real Time-PCR analysis showed that the VDR receptor gene expression in the cerebellum was increased significantly ($p<0.01$) in diabetic condition and it reversed to near control value in insulin and Vitamin D_3-treated diabetic rats (Table 4).

4. Discussion

Diabetic mellitus is a widespread disease that often results in a triad of pathology; neuropathy, retinopathy, and peripheral neuropathy. The mechanisms by which hyperglycemia causes neural degeneration is *via* the increased intracellular glucose that accompanies diabetes by which leads to altered neurotransmitter functions and reduced motor activity. Previous studies showed that pancreatic insulin secretion is inhibited by Vitamin D deficiency (Norman et al., 1980). An increased prevalence of diabetes has been associated with Vitamin D-deficient individuals (Chiu et al., 2004). Poorly controlled diabetes mellitus results in structural and functional changes in many brain regions. STZ-induced diabetic rats' cell death is increased and proliferation decreased in the cerebellum, indicating overall cell loss (Alfonso et al., 2006).

There is evidence that Vitamin D stimulate pancreatic insulin secretion directly. Vitamin D exerts its effects through nuclear Vitamin D receptors (Zeitz et al., 2003), which are found in a wide variety of tissues, including T and B lymphocytes, skeletal muscle, and the pancreatic islet β-cells (Walters, 1992). In individuals with diabetes mellitus, Vitamin D_3 treatment increase insulin secretion and improve glucose tolerance (Borissova et al., 2003 Rudnicki and Molsted-Pedersen, 1997). The facts' are increased blood glucose level and decreased body weight, observed during diabetes, are similar with previous reports as a result of the marked destruction of insulin secreting pancreatic β-cells by STZ (Junod et al., 1969). Vitamin D_3 and Insulin treatment normalized the increased blood glucose level and brought back the decreased body weight to control values.

Diabetes mellitus has been reported to be accompanied by a number of behavioral and hormonal abnormalities, including reduced locomotor activity (Marchall et al., 1976). Rota rod test has been used to examine the Motor in-coordination (Cendelin et al., 2008). The Rota rod experiment demonstrated the impairment of the motor function and coordination in the diabetic rats. Diabetes rats showed lower fall off time from the rotating rod when compared to control, suggesting impairment in their ability to integrate sensory input with appropriate motor commands to balance their posture. At the same time, they adjusted their limb movements on the metallic rod which is indicative of cerebellar dysfunction. The Vitamin D_3 and insulin-treated diabetic rats increased the fall off time from the rod compared to STZ-induced diabetic rats. Our findings indicate that Vitamin D_3 normalizes their alleviated stress level which assists in lowering their time for spatial recognition and thus helps to maintain their posture during movement on the rod.

The changes in muscarinic acetylcholine receptor have been implicated in the pathophysiology of many major diseases of the central nervous system. Earlier studies, from our laboratory have proved the functional regulation of the central neurotransmitter receptor subtypes during diabetes, pancreatic regeneration, cell proliferation and insulin secretion (Paulose et al., 1988; Sudha and Paulose, 1998; Abraham and Paulose, 1999; Biju et al., 2001; Mohanan et al., 2005; Kaimal et al., 2007; Gireesh et al., 2008). Gene expression studies show that the mRNA level of muscarinic M1, M3 receptors and acetylcholine esterase in the cerebellum were substantially increased when compared to control. Binding parameters B_{max} of total muscarinic, muscarinic M1 and M3 receptor was increased in diabetic rats compared to control. Earlier reports showed significant alterations in neurotransmitters during hyperglycaemia and causes degenerative changes in neurons of the central nervous system (Garris, 1990; Lackovic et al., 1990; Bhardwaj et al., 1999). It is hypothesized that the cerebellum participates in the learning and coordination of anticipatory operations which are necessary for the effective and timely directing of cognitive and non-cognitive resources (Allen et al., 1997). The current study reveals the anti-diabetic function of Vitamin D_3 and insulin on muscarinic M1, M3 receptors and acetylcholine esterase by normalizing the altered receptor gene expression and binding parameters to near control.

The present research reveals a major increase in α7 nicotinic receptor gene expression in the cerebellum of STZ-induced diabetes rats. Neuronal nicotinic cholinergic receptors are crucial to acetylcholine neurotransmission in both the CNS and autonomic nervous system. However, in the CNS, these receptors are more often associated with modulation of release of several neurotransmitters including dopamine, norepinephrine, GABA and glutamate (Wonnacott, 1997; Girod and Role, 2001). In the cerebellum, nicotinic acetylcholine receptors mediate the release of glutamate (Reno et al., 2004; De Filippi et al., 2001; Rossi et al., 2003) and norepinephrine (O'Leary and Leslie, 2003). Thus, these receptors significantly influence the activity within the cerebellar circuitry and deregulation of this activity could contribute to diabetes mellitus associated disorders involving the cerebellum. Abnormalities of nicotinic acetylcholine receptor function in the hippocampus lead to cognitive and memory impairments (Green et al., 2005; Levin et al., 2002) and sensory gating deficits (Adler et al., 1998). Vitamin D_3 supplementation proved a beneficial effect in standardising the altered gene expression to near control stage. Also, insulin treatment showed no significant effect in the gene expression level of STZ-induced diabetic rats.

Dopamine is one of the principal neurotransmitters in major neural systems of the brain (Fallon and Moore, 1978; Lindvall and Bjorklund, 1983). Furthermore, many behavioral studies have shown evidence that the mesolimbic dopamine system plays an important role in regulating exploratory and locomotor behavior (Fink and Smith, 1979; Funada et al., 1994). Dopamine D1 and D2 receptors subtypes have been suggested to mediate behavior responses. Antagonists of D1 and D2 receptors have been shown to block several well-characterized behaviors, including locomotor hyperactivity (Koob, 1992a,b). Activation of dopamine D1 receptors is required for the full expression of D2 dopamine receptor-mediated behavioral responses in normal animals (Dreher and Jackson, 1989; Logoni et al., 1987). Haloperidol and SCH23390, a selective dopamine D1 receptor antagonist, significantly reduced spontaneous locomotor activity in diabetic mice, but not in nondiabetic mice (Kamei et al., 1994). Our data showed a significant down-regulation of dopamine D1 receptor and an up-regulation of dopamine D2 receptor in the diabetes rats' cerebellum. Hyperglycemia during diabetes is reported to damage dopaminergic functions, as shown by changes in dopamine metabolism in the human brain and the brains of animals with experimentally induced diabetes (Lozovsky et al., 1981; Serri et al., 1985; Laokovic et al., 1990). Also the binding parameter of total dopamine receptor showed a decrease in STZ-diabetes and all the altered parameter were reversed to near control by the treatment with Vitamin D_3 and insulin.

Glucose plays a critical role as an energy source in the functioning of brain. For utilizing glucose in brain, it must be transported initially through the walls of cerebral blood vessels and subsequently through the plasma membranes of glial cells and neurons. GLUT3 had its

highest expression in brain and neural tissue hence being called the brain glucose transporter (Gould and Holman, 1993). Alterations in glucose utilization are known to occur in the important regions of brain connected with learning and memory (Krebs and Parent, 2005; McNay et al., 2000). Operant conditioning training would induce an increase in GLUT1and GLUT3 expression in memory-related structures in brain (Choeiri et al., 2005). The mRNA level of GLUT3 was up-regulated significantly in the cerebellum of STZ-induced diabetic rats. This indicates imbalanced glucose transport in the neurons of cerebellum. The Vitamin D_3 and insulin treatment stabilised the glucose transport mechanism mediated through GLUT3 in the cerebellum of STZ-induced diabetic rats.

In this study the altered expression of insulin receptor in the cerebellum of diabetic rat was brought back to near control level by the treatment with Vitamin D_3 and insulin. The memory-improving effect of glucose was shown by Lapp (1981). Experiments have shown the ability of small doses of insulin (0.4–0.8 U/kg) to reverse the amnesia produced by a 2 mg/kg scopolamine injection (Blanchard and Duncan, 1997; Messier and Destrade, 1994) and intra-cerebroventricular injection of insulin facilitates memory (Park et al., 1968). The wide distribution of insulin and insulin receptors in the brain as well as the presence of insulin-dependent glucose transporters suggests that insulin in the brain participates in several cognitive functions, including learning and memory. An obvious problem that has impeded further research is that exogenous insulin injection can reduce blood glucose and lead to hypoglycaemia which is associated with impaired memory (Kopf and Baratti, 1995; Kopf et al., 1998; Santucci et al., 1990). Cognitive impairments associated with diabetes mellitus caused by inadequate insulin/insulin receptor functions have also been documented.

Evidence suggests that Vitamin D_3 has potential benefits with respect to diabetes. Cholecalciferol has been shown lower blood pressure (Vianna et al., 1992) and may have a role on normal pancreatic function and treatment of diabetes (Bland et al., 2004). VDR is expressed in most brain areas. Vitamin D_3, has been detected in the cerebrospinal fluid, and this hormone has been shown to cross the blood-brain barrier (Balabanova et al., 1984). The presence of VDR in the limbic system, cortex, cerebellum of rodents and humans (Eyles et al., 2005; Musiol et al., 1992) support a functional role for Vitamin D_3 in the regulation of behavior and cognitive functions. Studies have shown that Vitamin D_3 confers regulatory benefits in neuronal Ca++ homeostasis and protects neurons from excess calcium entry in the brain (Brewer et al., 2001).Our result showed an increased expression of VDR in the cerebellum of diabetic rats. A previous study with RT-PCR on RNA extracts from the cerebellum, spinal cord, thalamus and whole brain showed the presence of VDR encoding transcripts, indicating VDR expression in these areas (Veenstra et al., 1998).Vitamin D_3 supplementation and insulin treatment normalizes the increased expression of VDR in diabetic rats to near control.

The direct glucose toxicity in neurons is caused by increased intracellular glucose oxidation (Nishikawa et al., 2000). This leads to an increase in production of reactive species in diabetic rats to play a central role in neuronal damage. The cerebellum has generally been suggested to be involved in the control and integration of motor processes, as well as cognitive functions. In the current study, we observed the neuroprotective effect of Vitamin D_3 on cholinergic receptors, insulin receptors, acetylcholine esterase and GLUT3 in cerebellum, which is responsible for the coordination of voluntary motor movement, balance and equilibrium and declarative memory. The results of this study have demonstrated that the supplementation of Vitamin D_3 to STZ-induced diabetic rats has beneficial effects in reducing the alterations in cholinergic receptors and imbalanced glucose utilization in cerebellum. These results unravelled the therapeutic effect of Vitamin D3 supplementation.

5. Conclusion

Treatment of diabetes mellitus is complex, requiring multifaceted lifestyle change and, for many, self-regulation of insulin levels in the blood. Uncontrolled hyperglycaemia, deficiencies of central insulin, or both contributes to cerebellar disorders mediated with cholinergic neurons. Vitamin D_3 exhibited a potential effect in improving glucose homeostasis and reversing the altered functional regulation of cholinergic, dopaminergic, insulin and Vitamin D receptors, acetylcholine esterase and GLUT3 activity in the cerebellum of STZ-induced diabetic rats. These results provide a confirmatory evidence for neuroprotective role of Vitamin D_3 and represent a novel possibility for the better management of diabetic mediated neurological complications.

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