Vitamin D protects diabetic rats from neuropathic changes by improving insulin sensitivity and upregulating vitamin D receptors

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Background
There is emerging evidence of neuroprotective roles for vitamin D. However, its role in the pathogenesis of type 2 diabetes mellitus (T2DM) and its exact mechanism of action in neuroprotection are still unclear. The present work was designed to examine the effect of vitamin D supplementation on insulin sensitivity and nerve conduction velocity with and without insulin treatment in a diabetic model.

Materials and methods
This study was carried out on 50 male adult rats. They were divided into five groups: a control group, a diabetic group, in which T2DM was induced; a diabetic insulin-treated group, in which diabetic rats were treated with insulin alone; a diabetic vitamin D-treated group, in which diabetic rats were treated with vitamin D alone; and finally, a diabetic with combined insulin and vitamin D treatment group. At the end of the experimental period, blood samples were obtained from all animals for measurement of serum glucose and insulin, together with the oxidative stress marker malondialdehyde (MDA) and inflammatory markers interleukins 1β and 10 (IL1β and IL10). Nerve conduction velocity was performed on a dissected sciatic nerve. In addition, vitamin D receptor (VDR) gene expressions in pancreatic (*VDR-P*) and sciatic nerve (*VDR-N*) tissues were estimated and the homeostasis model assessment for insulin resistance index was calculated for each group.

Results
Data showed a significant reduction in nerve conduction velocity of the sciatic nerve, together with increased insulin resistance in diabetic rats that paralleled increased MDA and IL1β and decreased IL10. Administration of insulin alone, vitamin D alone, or both combined after induction of diabetes improved the nerve conduction velocity. This improvement was accompanied by significant enhancement of *VDR-P* and *VDR-N* gene expression, together with reduction in oxidative stress and inflammatory state.

Conclusion
The improvement of insulin sensitivity and neuroprotection with vitamin D supplementation in T2DM is related to restoration of *VDR-P* and *VDR-N* expression. Thus, vitamin D could be a novel approach to lower neuropathic risk in diabetes.

Keywords:
1,25-dihydroxyvitamin D3, insulin resistance, nerve conduction velocity, type 2 diabetes mellitus, vitamin D receptor

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Introduction

Type 2 diabetes mellitus (T2DM) is characterized by peripheral insulin resistance and pancreatic b-cell dysfunction. It is prevalent worldwide, with significant comorbidity and mortality because of microvascular and macrovascular complications [1].

There is accumulating evidence suggesting that vitamin D status plays a role in many nonskeletal functions including diabetes mellitus (DM) [2,3].

Vitamin D deficiency and insufficiency have been defined as a 25-hydroxyvitamin D [25(OH)D] less than 20 ng/ml and 21–29 ng/ml, respectively. For every 100 IU of vitamin D ingested, the blood level of 25(OH)D increases by ~1 ng/ml. It is estimated that children need at least 400–1000 IU of vitamin D a day, whereas teenagers and adults need at least 2000 IU of vitamin D a day to fulfill their body's vitamin D requirement. It is estimated that one billion individuals worldwide are vitamin D deficient or insufficient. Correcting and preventing this deficiency

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could have an enormous impact on reducing health costs worldwide [4].

Vitamin D is a secosteroid that is obtained from dietary sources, either food or supplements, and exposure to sunlight. It needs to be hydroxylated twice to become biologically active. Vitamin D is transported to the liver, where it is first hydroxylated by 25-hydroxylase into 25(OH)D. This is the major circulating form used as an indicator of vitamin D status. The second hydroxylation occurs in the kidney by 1α-hydroxylase, a product of the CYP27B1. Here, the largest amount of the biologically active form of vitamin D, 1,25-dihydroxyvitamin D [1,25(OH)2D], is formed [5]. To date, only a few clinical trials examining the relation between vitamin D deficiency with glycemic control as primary outcome in T2DM have been performed [6]. The results of these clinical trials are inconsistent, mostly because of the small sample size, low dose of vitamin D supplementation, and short duration of the trials.

Vitamin D exerts its effects through its genomic and nongenomic responses. The nuclear vitamin D receptor (VDR) is a member of the thyroid hormone and retinoic acid receptor subfamily of nuclear hormone receptors that heterodimerizes with retinoid X receptor (RXR) isoforms to regulate the expression of genes encoding factors, which, in a variety of cell types, control functions such as proliferation, differentiation, metabolism, ion transport, and apoptosis [7].

When the VDR is associated with plasma membrane caveolae, the secosteroid hormone can activate a variety of rapid response (RR) pathways that may include kinases, phosphatases, or ion channels. These signaling cascades can either alter gene expression through their cognate promoter element or they can regulate gene expression by using the VDR as a substrate. It has been reported previously that phosphorylation of the VDR can modulate genomic potency and efficacy [7]. Collectively, nongenomic signaling that affects gene expression is often termed cross-talk.

It is generally accepted from the X-ray structure of the VDR with its bound 1α,25(OH)2D3 that it possesses a single ligand-binding domain, with its ligand bound strictly in the 6-s-trans shape [8]. This model presents a paradox for VDR as a 6-s-trans shape of 1α,25(OH)2D3 is obligatory for genomic responses, whereas a 6-s-cis shape is required for RR.

Thus, a conundrum is posed as to how a receptor with one formal ligand-binding domain binds ligands of different shapes to generate two distinct biological outcomes.

One possible solution to this conundrum was derived from molecular mechanics docking of a vitamin D analogue to the VDR [9]. Silico computational work provided evidence that VDR contains an alternative ligand-binding pocket that can accommodate the natural hormone or analogous that are known to be agonists only for RR [9].

In addition, a conformational ensemble provides a mechanism by which the VDR can signal both genomic and RR. Mizwicki *et al*. [9,10] assert that the resolution of the conundrum stated above is a proposed receptor ensemble model that describes a mechanism whereby a classic steroid (nuclear) receptor accommodates differently shaped ligands to initiate either rapid or genomic responses. This model posits that unbound receptor macromolecules exist in the cytoplasm in multiple, equilibrating receptor conformations that adhere to the laws associated with standard statistical distributions. It should be noted that 1α,25(OH)2D3 is capable of altering its conformation much more quickly than the receptor protein, rendering the entire ensemble of 1α,25(OH)2D3 conformations able to sample each of the individual protein ensemble conformations [11].

On the basis of numerous studies, as many as 500–1000 genes may be modulated by VDR ligands [12]. Many of these effects on gene expression are primary in that they involve direct VDR–RXR binding to vitamin D-responsive elements in or near the genes in question, leading to the concept of a 'vitamin D receptor cistrome' analogous to the estrogen receptor α cistrome mapped by Lupien *et al*. [13]. VDR is expressed in many tissues and cell types including the brain, the vascular system, numerous endocrine organs, the immune system, and muscle [12], plus the existence of many extrarenal sites of CYP27B1 expression [14] to catalyze local 1,25(OH)2D production.

The microvascular complications of diabetes carry a high morbidity [15]. The most common microvascular complication is neuropathy. The incidence of neuropathy increases with the duration of diabetes and is accelerated by poor control [16].

Oxidative stress is associated with the development of apoptosis in neurons and supporting glial cells and could thus be the unifying mechanism that leads to nervous system damage in diabetes [17,18].

Neurons are not only lost in diabetes, but their ability to regenerate is also impaired, particularly the small-caliber nerve fibers [19]. In patients with diabetic neuropathy, both degeneration and regeneration are present simultaneously, suggesting that the disorder is

highly dynamic [20]. Over time, the balance between degeneration and regeneration shifts toward more degeneration [19].

The mechanisms leading to loss of regeneration may include impaired insulin action [21], loss of growth factor systems [22], and decrease in specific isoforms of protein kinase C [23]. Schwann cells are important in the regenerative process, and these can also be impaired in diabetes through hyperglycemia, hypoxia, and oxidative stress [24].

The aim of our work was to examine the modulation of vitamin D on insulin sensitivity and nerve conduction (NC) velocity in adult diabetic male rats, its interaction with insulin treatment, and to clarify the underlying mechanisms involved in this effect.

Materials and methods

The experiments were conducted in accordance with the ethical guidelines for investigations of laboratory animals and were approved by the Committee of Physiology Department, Faculty of Medicine, and Cairo University.

Experimental animals

Fifty male albino rats weighing about 150–200 g were used as experimental animals in the present investigation. They were obtained from the animal house of the National Research Center (Giza, Egypt). All rats were housed for 1 week before a diet intervention. The chosen animals were housed in plastic well-aerated cages at normal atmospheric temperature (22 ± 3°C) as well as a normal light/dark cycle. Moreover, they were allowed access of water and supplied daily with a standard diet of known composition.

Ten animals were selected randomly as a normal control group (group 1), which was fed with standard rodent diet (6.5% kcal fat). The remaining rats were used to establish T2DM models. They were fed with a high-fat diet (60% kcal fat) for 2 weeks. Then, these rats were administered an intraperitoneal injection of two low doses of streptozotocin (30 mg/kg in 0.01 mol/l citrate buffer; Sigma, St. Louis, Missouri, USA) at an interval of 24 h [25]. Three days later, T2DM was confirmed in high-fat diet rats by measuring fasting serum glucose and insulin. These DM model rats were randomly divided as follows:

Group 2 (DM group): This group was maintained on usual care and was left untreated, but received a saline injection in equal volume to that of treated rats until the end of the study.

Group 3 (INS group): This group received neutral protamine hagedorn (NPH) human insulin at a dose of (1 U) by a subcutaneous injection twice daily between 8:00–9:00 and 16:00–17:00 h for 6 weeks [26].

Group 4 (vitamin D group): This group was administered a vitamin D injection; doxercalciferol was administered intraperitoneally at 150 ng three times a week for 6 weeks [27].

Group 5 (INS+vitamin D group): T2DM rats were treated with combined insulin and vitamin D 48 h after a streptozotocin injection (day 2) and confirmation of DM at the same doses administered to groups 3 and 4, respectively, for 6 weeks.

At the end of the treatment intervention period, blood samples were drawn from the retro-orbital vein and the serum samples were separated by centrifuge. Then, the rats were killed by cervical dislocation. The entire pancreas and left sciatic nerve tissue were dissected and kept frozen at –80°C in liquid nitrogen until used for assessment of gene expression of the VDR. The dissected right sciatic nerves were used for assessment of the NC velocity using the PowerLab Data Acquisition System (AD Instruments, Castle Hill, Australia).

The following biochemical parameters were assessed in the serum: glucose, insulin, oxidative stress marker malondialdehyde (MDA), and the inflammatory markers interleukins 1β and 10 (IL1β and IL10).

Nerve conduction velocity measurements

Electrophysiological recording

The dissected sciatic nerve was carried in a nerve chamber designed for recording of action potential from the isolated nerve. It has 15 stainless-wire electrodes. The nerve was dissected free without any muscle residue. About 2 cm of the nerve was positioned over the electrodes and embedded in paraffin oil to maximize signal amplitude and prevent drying. The proximal part of the nerve was stimulated by two platinum stimulating hook electrodes and the recording electrode was placed 1–2 cm apart from the stimulating one.

Electrophysiological measurements were performed using an AD Instruments PowerLab 4/25 Stimulator and Bio AMP Amplifier (Castle Hill, Australia), followed by a computer-assisted data analysis. Sciatic nerves were stimulated with square wave pulses of 200 μs duration at 1–10 V for conduction velocities. Conduction velocity is measured by dividing the distance between the stimulating and recording electrodes by the latent period, which is the time elapsed between the application of stimulus until the peak of the maximum compound action potential [28].

Biochemical measurements

Measurement of fasting plasma glucose level

Plasma glucose in blood samples was measured using the oxidase–peroxidase method [29].

Measurement of plasma insulin

Plasma insulin levels were analyzed using enzyme-linked immunosorbent assay (Dako, Carpinteria, California, USA) according to the manufacturer's instructions [30].

Homeostasis model assessment for insulin resistance test

To estimate insulin resistance, the homeostasis model assessment for insulin resistance (HOMA-IR) (insulin resistance index) [31] was used, calculated as the product of fasting insulin (μIU) and fasting glucose (mmol/l) divided by 22.5. A lower index indicates greater insulin sensitivity.

Measurement of malondialdehyde

To measure the MDA concentration, 100 mg of tissue in 1 ml PBS (pH 7.0) was homogenized with a micropestle in a microtube [32]. Twenty percent trichloroacetic acid (TCA) was added to tissue homogenate to precipitate the protein and centrifuged. Supernatants were collected and thiobarbituric acid solution was added to the supernatants. After boiling for 10 min in a water bath, the absorbance was measured. The concentration of MDA was calculated using the standard curve.

Measurement of interleukin 1β and interleukin 10

IL1b and IL10 were measured using enzyme-linked immunosorbent assay (Quantikine R&D System, Minneapolis, USA) according to the manufacturer's instructions.

Detection of *VDR* gene expression by quantitative real-time polymerase chain reaction

RNA isolation and reverse transcription

RNA was extracted from tissues homogenate using a Mini RNA Isolation Kit (Zymo Research, Orange, California, USA) according to the manufacturer's instructions. The RNA concentration was determined spectrophotometrically at 260 nm using the NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and RNA purity was checked by means of the absorbance ratio at 260/280 nm. RNA integrity was assessed by electrophoresis on 2% agarose gels. One microgram of RNA was used in the subsequent cDNA synthesis reaction, which was performed using the Reverse Transcription System (Promega, Leiden, the Netherlands). Total RNA was incubated at 70°C for 10 min to prevent secondary structures. The RNA was supplemented with $MgCl_2$ (25 mmol/l), RT buffer (10´), dNTP mixture (10 mmol/l), oligo(dT) primers, RNase inhibitor (20 U), and avain myeloblastosis virus (AMV) reverse transcriptase (20 U/μl). This mixture was incubated at 42°C for 1 h.

Quantitative real-time polymerase chain reaction

qPCR was performed in an optical 96-well plate using an ABI PRISM 7500 Fast Sequence Detection System (Applied Biosystems, Carlsbad, California, USA) and universal cycling conditions (10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C). The reaction contained SYBR Green Master Mix (Applied Biosystems), gene-specific forward and reverse primers (10 μmol/l), cDNA, and nuclease-free water. The sequences of PCR primer pairs used are shown in Table 1. Data were analyzed using the ABI PRISM sequence detection system software and quantified using the v1·7 Sequence Detection Software (PE Biosystems, Foster City, California, USA). The relative expression of the studied genes was calculated using the comparative threshold cycle method. All values were normalized to the *GAPDH*, which was used as the control housekeeping gene [33].

Statistical analysis

The results were analyzed using SPSS computer software package (version 21; IBM, New York, USA). Data were presented as mean ± SD. Comparison of quantitative variables between the studied groups was performed using the Kruskal–Wallis test with the Wilcoxon signed-rank test according to the result of the Shapiro–Wilk test for normality of distribution. Correlations were calculated using Spearman's test. Results were considered statistically significant at *P* value 0.05 or less.

Results

The levels of serum fasting glucose, fasting insulin (μIU/l), and homeostatic model assessment index

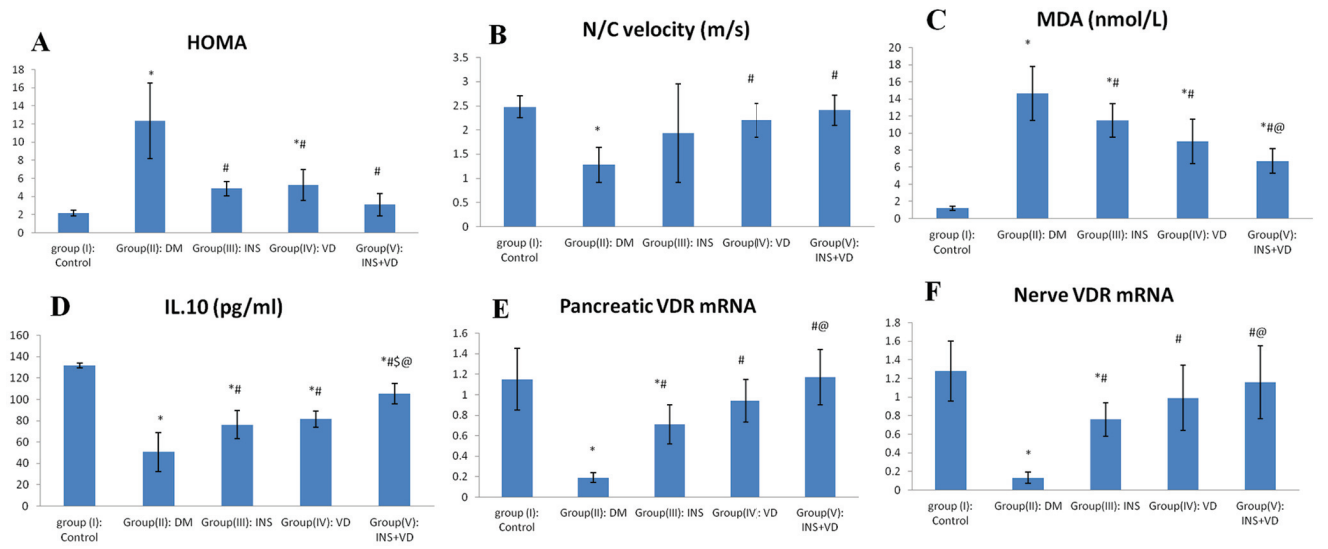
The levels of serum fasting glucose, fasting insulin (μIU/l), and homeostatic model assessment index

Table 1 Primer sequences used for real-time polymerase chain reaction

Primers	Sequence
VDR	Forward: 5´-GATGC TGGTG CTGAG TATGT CG-3´ Reverse: 5´-GTGGT GCAGG ATGCA TTGCT CTGA-3´
GAPDH	Forward: 5´-GATGC TGGTG CTGAG TATGT CG-3´ Reverse: 5´-GTGGTGCAGGATGCATTGCTCTGA-3´

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; VDR, vitamin D receptor.

Figure 1



The effect of insulin, vitamin D treatment alone, or combined on homeostasis model assessment (HOMA) (a), nerve conduction (NC) velocity (b), the serum malondialdehyde (MDA) (c), interleukin 10 (IL10) (d), pancreatic vitamin D receptor (VDR) mRNA (e), and nerve VDR mRNA (f) in a rat model of type 2 diabetes mellitus (mean ± SD) ($n = 10$ in each group). $^{*}P < 0.05$, statistically significant compared with the corresponding value in group 1; $^{\#}P < 0.05$, statistically significant compared with the corresponding value in group 2; $^{@}P < 0.05$, statistically significant compared with the corresponding value in group 3; $^{\$}P < 0.05$, statistically significant compared with the corresponding value in group 4.

Table 2 Mean ± SD values of serum fasting glucose (mg/dl), serum fasting insulin (μIU/l), homeostatic model assessment index, nerve conduction velocity (m/s), serum malondialdehyde (nmol/l), interleukins 1β and 10 (pg/ml), and the expression of pancreatic and nerve vitamin D receptor mRNA in the studied groups (n = 10 in each group)

Parameters	Group 1: control	Group 2: DM	Group 3: INS	Group 4: VD	Group 5: INS+VD
Glucose (mmol/l)	5.25 ± 0.56	13.96 ± 2.86*	7.09 ± 0.98#	8.87 ± 1.04*#	5.18 ± 1.97#$
Insulin (μIU/l)	9.31 ± 0.82	19.63 ± 3.70*	15.63 ± 2.77*#	13.15 ± 3.23*#	13.49 ± 2.25*#
HOMA	2.17 ± 0.31	12.35 ± 4.15*	4.87 ± 0.79#	5.25 ± 1.71*#	3.10 ± 1.25#
NC velocity (m/s)	2.48 ± 0.23	1.28 ± 0.36*	1.93 ± 1.02	2.20 ± 0.35#	2.41 ± 0.31#
MDA (nmol/l)	1.21 ± 0.25	14.64 ± 3.19*	11.49 ± 1.97*#	9.02 ± 2.59*#	6.73 ± 1.44*#@
IL1β (pg/ml)	30.92 ± 6.14	108.34 ± 12.05*	84.56 ± 11.80*#	83.27 ± 8.28*#	56.10 ± 10.85*#$@
IL10 (pg/ml)	131.59 ± 2.15	50.53 ± 18.14*	76.26 ± 13.21*#	81.42 ± 7.73*#	105.24 ± 9.56*#$@
Pancreatic VDR mRNA	1.15 ± 0.30	0.19 ± 0.05*	0.71 ± 0.19*#	0.94 ± 0.21#	1.17 ± 0.27#@
Nerve VDR mRNA	1.28 ± 0.32	0.13 ± 0.06*	0.76 ± 0.18*#	0.99 ± 0.35#	1.16 ± 0.39#@

DM, diabetes mellitus; HOMA, homeostasis model assessment; IL, interleukin; MDA, malondialdehyde; NC, nerve conduction; VD, vitamin D; VDR, vitamin D receptor; $^{*}P < 0.05$, statistically significant compared with the corresponding value in group 1; $^{\#}P < 0.05$, statistically significant compared with the corresponding value in group 2; $^{@}P < 0.05$, statistically significant compared with the corresponding value in group 3; $^{\$}P < 0.05$, statistically significant compared with the corresponding value in group 4.

are summarized in Table 2 and Fig. 1a. There was a statistically significant increase in serum glucose, insulin, and HOMA in diabetic rats ($P < 0.05$) compared with the control group. However, treatment with insulin or vitamin D either alone or combined produced comparable effects in reducing these parameters significantly compared with the diabetic rats. Although the combined treatment produced better improvement in insulin sensitivity compared with either treatment alone, it was statistically insignificant.

The results of nerve conduction velocity in the experimental groups

The results of NC velocity in the experimental groups are summarized in Table 2 and Fig. 1b. NC velocity was significantly decreased in the diabetic group ($P < 0.05$) compared with the control group, whereas it was significantly improved under vitamin D treatment in both groups 4 and 5, in contrast to insulin treatment, which was not associated with a significant improvement in the NC velocity; however, it also appeared insignificant compared with the control group, which showed a minor improvement in the NC velocity under insulin therapy.

The changes in the level of oxidative marker malondialdehyde and inflammatory markers interleukin 1β and interleukin 10

The changes in the level of oxidative marker MDA and inflammatory markers IL1β and IL10 are shown

in Table 2 and Fig. 1c and d. There was a significant increase in MDA (P < 0.05) together with the proinflammatory marker IL1β (P < 0.05) in the diabetic rats compared with the control animals, in association with a significant reduction in the anti-inflammatory marker IL10 (P < 0.05).

In terms of the effect of treatment on oxidative stress and inflammatory cytokines, there was a significant reduction in MDA levels (P < 0.05), either under insulin or vitamin D therapy alone, compared with the diabetic rats. Interestingly, there was a further significant reduction under combined treatment compared with insulin-treated rats alone (P < 0.05). These changes in the MDA parallel the significant decrease in IL1β either under insulin or vitamin D therapy alone compared with the diabetic rats. In addition, there was a further significant reduction under combined treatment compared with insulin-treated (P < 0.05) or vitamin D-treated rats alone (P < 0.05). However, there was a significant improvement in IL10 in groups 3, 4, and 5 (P < 0.05) compared with the diabetic rats, whereas combined treatment produced a significant improvement (P < 0.05) more than either treatment alone. This indicates the presence of synergistic effects between vitamin D and insulin therapy in reducing the production of inflammatory cytokines in diabetic rats.

The changes in the level of mRNA expression of pancreatic and nerve *VDR* in studied groups

The changes in the level of mRNA expression of pancreatic and nerve *VDR* in studied groups are summarized in Table 2 and Fig. 1e and f. The diabetic rats showed a statistically significant decrease (P < 0.05) in pancreatic *VDR* gene expression compared with the control group. There was a significant increase in both insulin-treated and vitamin D-treated rats (P < 0.05) compared with the diabetic rats, but they became comparable with that of the control group in groups 4 and 5 (P > 0.05). It was noted that the combined treatment produced a significant improvement from insulin treatment alone on the pancreatic *VDR* gene expression (P < 0.05).

In addition, there was a significant decrease in the gene expression of the *VDR* (P < 0.05) in the sciatic nerve of diabetic rats compared with the control group. However, treatment with insulin or vitamin D, each alone or combined, all caused significant rise in sciatic nerve *VDR* gene expression compared with the diabetic animals (P < 0.05), yet it was still higher than the control level (P < 0.05) in group 3, but it was comparable with the control in groups 4 and 5.

A precise analysis of the percentage of changes in the studied parameters different in our work indicates evidence for the presence of synergistic effects between vitamin D and insulin therapy (Table 3).

The results of the current study showed an inverse correlation between the level of insulin and NC velocity in all groups (r = −0.502 and P < 0.001) (Fig. 2a). However, this correlation was insignificant in group 3 (r = 0.132 and P = 0.717). However, there was a significant positive correlation between the gene expression of *VDR* in the sciatic nerve and NC velocity in all groups (r = 0.570 and P < 0.001) (Fig. 2b).

Discussion

The main defects that determine the development of T2DM are insulin resistance, pancreatic β-cell dysfunction, and systemic inflammation [34].

In the present work, improvements in insulin sensitivity and NC velocity with vitamin D supplementation were observed in a rat model of T2DM. There was also an enhancement in *VDR* gene expression in the pancreatic and the nerve tissue, together with amelioration of antioxidant and anti-inflammatory capacity.

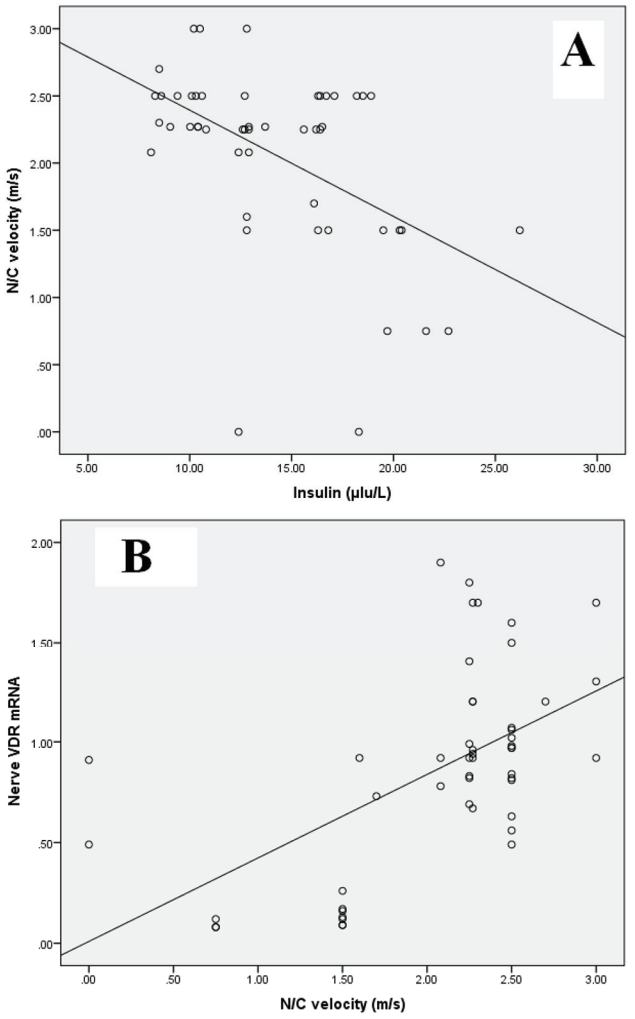
Our study showed that vitamin D treatment improved hyperglycemia, hyperinsulinemia, and insulin sensitivity

Table 3 Percentage changes in all parameters in the studied groups (n = 10 in each group)

Parameters	% change between groups 1 and 2	% change between groups 2 and 3	% change between groups 2 and 4	% change between groups 2 and 5
Glucose (mmol/l)	165.9	−49.21	−36.46	−62.89
Insulin (μIU/l)	110.85	−20.38	−33.01	−31.28
HOMA	469.12	−60.57	−57.49	−74.9
NC velocity (m/s)	−48.39	50.78	71.88	88.28
MDA (nmol/l)	1109.92	−21.52	−38.39	−54.03
IL1β (pg/ml)	250.39	−21.95	−23.14	−48.22
IL10 (pg/ml)	−61.6	50.92	61.13	108.27
Pancreatic *VDR* mRNA	−83.48	273.68	394.74	515.79
Nerve *VDR* mRNA	−89.84	484.62	661.54	792.31

HOMA, homeostasis model assessment; IL, interleukin; MDA, malondialdehyde; NC, nerve conduction; VD, vitamin D; VDR, vitamin D receptor.

Figure 2



Correlation between insulin and nerve conduction (NC) velocity (a) and correlation between nerve conduction velocity and *VDR* gene expression in the nerve (b) in all groups. VDR, vitamin D receptor.

significantly compared with the nontreated diabetic rats. The reduction in glucose level was found to be significantly higher in combined vitamin D with insulin therapy compared with insulin treatment alone. The improvement with vitamin D treatment was in agreement with the study of Norman *et al.* [35]. Others provided evidence that vitamin D supplementation exerted beneficial effects in obese spontaneously hypertensive rats and Wistar rats, where there was a reduction in glucose levels in vitamin D-supplemented animals [35–37]. It was found that short-term vitamin D replenishment in a Bangladeshi Asian population increased insulin secretion without altering glycemia, whereas longer vitamin D treatment also improved glucose levels [38].

The potential mechanisms by which vitamin D can affect glucose metabolism could be the result of a rapid nongenomic effect or a slower genomic effect of vitamin D through stimulation of insulin release by the increased expression of *VDR* [2]. This was in agreement with our observation that *VDR* pancreatic gene expression was enhanced in vitamin D-treated rats by binding of the 1,25(OH)2D–VDR complex to the vitamin D response element of the insulin receptor at the tissue level, enhancing insulin responsiveness for glucose transport [5].

Another possible mechanism is the suppression of the release of proinflammatory cytokines that are believed to mediate insulin resistance [2,5]. The latter hypothesis is supported by studies showing an association between low serum 25(OH)D and increased C-reactive protein levels [6]. This is in agreement with our finding of improvement in the anti-inflammatory marker IL10 and reduction in the proinflammatory marker IL1β with vitamin D therapy alone or combined with insulin. This improvement was significantly more in combined therapy than with either treatment alone.

In addition, vitamin D may indirectly influence the extracellular and intracellular calcium regulation, which is essential in mediating glucose transport in target tissues [39]. In an effort to understand the role of vitamin D in β-cell function, Nyomba *et al.* [40] found that in streptozotocin-induced diabetic rats, plasma calcium levels, vitamin D binding protein (DBP), circulating vitamin D, and bone mass were reduced. These defects have been attributed to altered vitamin D metabolism owing to an inhibitory effect of insulin deficiency on the activity of the renal 25(OH) D3 1α-hydroxylase [41].

Lee *et al.* [42] reported that osteocalcin, a bone-secreted hormone, also known as 'bone γ-carboxyglutamic acid protein (BGP)' improved insulin release from pancreatic β-cells and increased insulin metabolic responsiveness in target tissues. Interestingly, *BGP* is a gene classically induced by 1,25(OH)2D in osteoblasts, particularly in rats and humans [43,44].

Thus, vitamin D-induced bone osteocalcin, by supporting insulin release and action, could be considered an important adjunct in insuring glucose control to delay or lower the risk of advanced glycemic end products formation, characteristic of uncontrolled DM, which elicits microvascular and macrovascular complications in both type 1 and type 2 diabetes [45].

The current study showed that there was a significant increase in the levels of MDA in diabetic rats compared with the control group. Our finding that vitamin D treatment significantly suppressed oxidative stress was in agreement with the finding of Dong *et al.* [46].

The present data showed that there was a significant improvement in the NC velocity under vitamin D treatment, which was accompanied by an improvement in oxidative stress.

Evidence is presented to support the idea that both chronic and acute hyperglycemia cause oxidative stress in the peripheral nervous system, which can promote the development of diabetic neuropathy. Proteins that are damaged by oxidative stress have decreased biological activity, leading to loss of energy metabolism, cell signaling, transport, and ultimately, cell death. Examination of the data from animal and cell culture models of diabetes, as well as clinical trials of antioxidants, strongly implicates hyperglycemia-induced oxidative stress in diabetic neuropathy [47]. Thus, we can suppose that vitamin D, by improving the antioxidant capacity, plays an important role in the prevention of diabetic neuropathy.

Various studies have shown that 1,25(OH)2D3 can act on cells of the nervous system by modulating the production of neurotrophins. For instance, the synthesis of the nerve growth factor [48], neurotrophin 3 [49], and glial cell line-derived neurotrophic factor [50] was upregulated by 1,25(OH)2D3. In several cases, stimulation of neurotrophins production by 1,25(OH)2D3 was correlated with a neuroprotective effect [51].

In addition to its influence on neurotrophin synthesis, 1,25(OH)2D3 could mediate its neuroprotective effects through the modulation of neuronal Ca^{2+} homeostasis. In support of this hypothesis is the recent report of downregulation of the L-type voltage-sensitive Ca^{2+} channel in hippocampal neurons in the presence of 1,25(OH)2D3, which has been correlated with a neuroprotective effect against excitotoxic insults [52]. Another way in which 1,25(OH)2D3 might mediate its neuroprotective effect is to induce the synthesis of Ca^{2+}-binding proteins, such as parvalbumin [53]. 1,25(OH)2D3 has also been reported to inhibit the synthesis of inducible nitric oxide synthase [54], an enzyme induced in central nervous system (CNS) neurons and non-neuronal cells during various insults or diseases, such as ischemia, Alzheimer's disease, and experimental autoimmune encephalomyelitis. 1,25(OH)2D3 has also been reported to upregulate γ-glutamyl transpeptidase activity and expression of the corresponding gene in rat brain [55]. Because γ-glutamyl transpeptidase is largely involved in the glutathione cycle of the brain in cross-talk between astrocytes and neurons [56], an increase in glutathione levels is linked to a neuroprotective effect of 1,25(OH)2D3.

The neuroprotective effect of calcitriol observed in the present study cannot be explained by direct scavenging of reactive oxygen species (ROS) as shown by the lack of a direct effect of calcitriol on the generation of free radicals by the cell-free HXXO reaction [57].

Instead, calcitriol exerts its effect through genomic regulation. This conclusion should be confirmed by further research. The genomic impact of calcitriol can be confirmed by the experiments with the mRNA synthesis inhibitor, actinomycin-D, in future studies.

A genomic action is consistent with the conclusion that the effects of calcitriol reported in the present study are because of an increase in *VDR* gene expression in the nerve tissue. Indeed, upon binding of and activation by vitamin D, VDR forms a heterodimer complex with the RXRs. The VDR–RXR complex can bind to specific DNA sequences, termed vitamin D-responsive elements, located in the promoter regions of various vitamin D-dependent genes [57].

Indeed, 1α,25(OH)2D3–VDR is anti-inflammatory by blunting NFκB [58] and COX2 [59]. Finally, 1α,25(OH)2D3–VDR induces FOXO3 [60], an important molecular player in preventing oxidative damage [61]. Also, 1,25(OH)2D–VDR controls the expression of osteopontin (SPP1), which encodes myelination [62].

It is clear that the underlying factor in the neurological disorders is increased oxidative stress substantiated by the findings that the protein side-chains are modified either directly by ROS or reactive nitrogen species or indirectly by the products of lipid peroxidation [63].

In fact, mitochondrial damage occurs because of excess formation of ROS or reactive nitrogen species. Hyperglycemia induces mitochondrial changes such as release of cytochrome *c*, activation of caspase 3, altered biogenesis, and fission, which all lead to a programmed cell death [64].

Conclusion

This study links the effects of the 1,25(OH)2D3 in improving insulin sensitivity and neuropathy in a T2DM model to restoration of pancreatic and nerve *VDR* expression. 1,25(OH)2D3 altered insulin resistance and neuropathic changes significantly, which may be because of its genomic effects in addition to its antioxidant and anti-inflammatory capacity. Thus, vitamin D could be a novel approach to lower neuropathic risk in diabetes.

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Conflicts of interest
There are no conflicts of interest.

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