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The Protective Effect of Calcitriol on Diabetic Neuropathy in STZ-induced Diabetic Rats

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Authors' contributions

This work was carried out in collaboration between all authors. Author HIAE managed the experimental process and literature searches. Author MMEB supervise the experimental work, review literature searches, perform statistical analysis of the data and wrote the first draft of the manuscript. Author WMM revised the paper critically for important intellectual content. Author MAM do the histopathological examination and analysis. Author EAEB did the biochemical analysis and data interpretation. Author AEAY revised the paper. Author AHO designed the study, wrote the protocol and revised the paper. All authors read and approved the final manuscript.

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ABSTRACT

Diabetic peripheral neuropathy (DPN) is one of the diabetic complications that have a complex etiology and even with good glycemic control can have severe consequences. To date, there is no effective treatment for DPN. The current work aimed to investigate the possible protective effects of calcitriol on DPN by using streptozotocin-induced diabetic rats. Male rats were assigned into 5 equal groups: Normal control, diabetic untreated, diabetic treated with NPH Insulin, diabetic treated with calcitriol, diabetic received both insulin and calcitriol. Induction of diabetes mellitus was done

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by injection of a single dose of streptozotocin (30 mg/kg, intraperitonael). After 4 consecutive weeks, rats were subjected to tail flick latency test and blood samples were collected for biochemical assay of serum glucose, insulin, advanced glycation end products (AGEs). Sciatic nerves were dissected out for histopathological studies and estimation of tumor necrosis factor-α (TNF-α), superoxide dismutase (SOD) and malondialdhyde (MDA). Diabetes induced hyperalgesia, elevated levels of blood glucose, AGEs, TNF-α and MDA, decrease level of insulin and SOD together with marked structural changes in sciatic nerve. Minor to moderate improvement was recorded in either insulin- or calcitriol- treated diabetic subgroups. However, good amelioration was recorded in combination of insulin and calcitriol treatment. It is concluded that calcitriol improved the biochemical and histological changes induced by diabetes. Thus it may provide neural protection against DPN.

Keywords: DM; calcitriol; sciatic nerve; ultrastructure.

1. INTRODUCTION

Diabetes mellitus (DM) is considered a major cause of mortality and morbidity [1]. Diabetic peripheral neuropathy (DPN) is one of the most common diabetic complications with the risk of pain, foot ulcers and amputation [2]. There is no doubt that, hyperglycemia plays a key role in the development and progression of DPN as well as other microvascular complications of diabetes. Several mechanisms are thought to be involved in the pathogenesis of DPN such as increased polyolpathway flux, activation of protein kinase C (PKC), increased hexosamine pathway flux and increased advanced glycation end-products (AGEs) formation [3]. The formation of AGEs not only modifies protein properties, but also induces biological damage *In vivo* [4]. It is noteworthy that, enhanced oxidative stress causes alteration of nerve blood supply, nerve structure and endoneural metabolism that contribute to peripheral nerve damage and dysfunction in diabetic conditions [5]. It was hypothesized that oxidative stress is the trigger for the complications cascade involved in diabetic neuropathy. It is difficult to separate the effects of oxidative stress from changes due to AGE production because the latter produces reactive oxygen species (ROS) and is accelerated by ROS [6]. All the above considerations support the current hypothesis that, the proper therapeutic approach to prevent diabetic complications is to concentrate not only on early glycemic control but also on reducing factors related to oxidative stress and AGEs in particular [7].

Calcitriol, a fat-soluble vitamin, has a classical role in calcium and bone homeostasis beside other roles, through binding to vitamin D receptors (VDRs), in non-skeletal tissues as pancreas and other tissues [8]. It is remarkable that vitamin D deficiency may have negative effects on glucose intolerance, insulin secretion and T2DM [9], either directly via VDRs activation or indirectly via calcemic hormones and inflammation [10]. Furthermore, vitamin D has significant roles in the synthesis and release of insulin, as both 1-α-hydroxylase and VDRs are present in pancreatic β cells [11]. Hypovitaminosis D is highly prevalent in patients with Type 2 diabetes [12] and could be a prelude to the development of diabetic neuropathy via neurotrophic deficits [13]. Likewise, a wealth of evidences has suggested that adequate intake of vitamin D may prevent or at least delay the onset of diabetes, as well as its complications [14,15]. Diabetic neuropathic pain treatment is difficult because no specific relief medication is available, even the use of conventional lines of treatment for glycemic control does not prevent the progression of these complications. Aiming to assess the neuroprotective effects of calcitriol in DPN, we examined the possible protective effects of calcitriol in a rat model of STZ-induced type-I DM.

2. MATERIALS AND METHODS

2.1 Animals

Fifty male Sprague-Dawley rats of the same age, weighing 100-150 gm were used in the present experiments. All rats were housed in groups of five per cage in standard rat cages under controlled temperature (22-24°C), humidity (30-40%) and artificial light/dark cycle with free access to food and water ad libitum. Animals were acclimatized to these conditions for at least 1 week prior to the experiment. All animals received appropriate care in compliance with the Public Health Service Policy on Use of Laboratory Animals published by the National Institutes of Health and was approved by the Ethical Committee of the College of Medicine,

Menoufia University, Egypt. All the procedures described below were carried out in accordance with the guidelines of the EU Directive 2010/63/EU for animal experiments.

2.2 Chemicals

Streptozotocin (STZ) powder; Sigma–Aldrich; Calcitriol tablet or capsule (Rocaltrol 0.5 µg, Roche Co); Isophane insulin (NPH) (mixtard 40 IU vial, Novo Nordisk Co Denmark); Cholesterol powder; Sigma-Aldrich; Ursodeoxycholic acid tablet or capsule (livagoal 450 mg,Marcyrl CO Egypt); Sucrose (local source); dissolved lamp fat (local source); Citrate buffer (pH 4.4, 0.1 M,Biodiagnostic CO); Tris hydrochloric buffer (Biodiagnostic CO); Phosphate buffer (Biodiagnostic CO); 10% formalin solution (Sigma-Aldrich).

2.3 Methods

2.3.1 Establishment of rat model of type 2 diabetes

Rats were fed with high-fat and high-sugar diet (feed formula: 67% normal diet, 20% sucrose, 10% tried lard, 2% cholesterol, 1% bile salts) with free access to water for 6 weeks. Rat's weight was recorded daily throughout the whole 6 weeks period. Rats received a single dose daily of freshly dissolved STZ (30 mg/kg body weight, IP) in a pH 4.5 citric acid buffer. Following STZ injection, rats given drinking water supplemented with sucrose (15 g/L) for 48 h, to limit early mortality as stores of insulin are released from damaged pancreatic islets [16]. Fasting blood glucose level was measured using rat-tail vein blood by glucose oxidase reagent strips (MediSense blood glucose meter and strips) after the seventh day of STZ injection. The rats with FBG ≥126 mg/dl and/or 2hBG ≥200 mg/dl were considered diabetic and used in the experiment according to Ren et al. [17]. Ten rats were excluded from the study due to death or induction failure.

This study was designed to examine the possible protective effect of calcitriol with or without insulin against the development of DPN. The rats classified into five groups (8 rats for each group):

1. **Normal control group:** Non diabetic control vehicle group received an equal volume of vehicles in the form of single dose of citrate buffer IP and coconut oil by oral gavage, for four weeks.
2. **Diabetic untreated**: Received equal volume of coconut oil orally by gavage.
3. **Diabetic treated with insulin:** Received SC injection of (10 IU/kg/day) NPH insulin [18].
4. **Diabetic treated with calcitriol:** Received calcitriol orally by gavage (0.03 µg/kg/day) dissolved in coconut oil [17].
5. **Diabetic treated with calcitriol and insulin**: Received both calcitriol and insulin as in group 3, 4.

2.3.2 Tail flick test

Rats of all experimental groups were administered drugs for four weeks. At the end of the experiment the rats of each group subjected to the tail flick test. Tail flick test is one of the standard tests for measuring the rate of nociception. In this test, thermal light with the intensity of 5 is directed to the distal part of rat's tail by Tail flick instrument (Harvard apparatus, Inc.) and the time from the onset of stimulation to sudden tail's withdrawal (tail flick latency) was measured. In order to avoid tissue damage, the stimulus cut-off was set to the maximum of 10 seconds. Mean of three measures was recorded as tail flick latency. The interval of three measurements was 5 minutes [19].

2.3.3 Biochemical measurements

Venous blood samples were collected from the retro-orbital plexus of rats for biochemical assay. The samples were divided into two fractions: 1) One ml of blood into flouride tube for FBG (by enzymatic colorimetric test, using Spin react kit, SPAIN). 2) Plain tubes left for 30 minute then centrifuged for 10 minutes at 3000 rpm; sera were separated in several aliquots and stored at –20°C until assay of serum insulin using rat ELISA kit (DRG International, Inc., GERMANY), AGEs determined using commercially available rat ELISA kits following the instructions supplied by the manufacturer (G science, Inc., USA). Animals were then sacrificed and sciatic nerve specimens were immediately dissected out. Half of tissue samples were processed for preparation of tissue homogenate with 0.1 tris-HCL buffer (pH 7.4) then centrifuged and stored at -80°C till using the supernatant for estimation of tumor necrosis factor-α (TNF-α) as one of the inflammatory cytokines using commercially available rat ELISA kits following the instructions supplied by the manufacturer (eBioscience, Inc., USA) and colometric determination of superoxide dismutase (SOD) as one of the endogenous

antioxidants using kits supplied by Biodiagnostic, Inc, Egypt. The results were shown as pg/ml for TNF-α and unit/mg tissue protein for SOD. Malondialdhyde (MDA) levels, as one of the lipid peroxidation product, were estimated by the double heating method of the supernatant of sciatic nerve tissue homogenate using commercially Biodiagnostic, Inc, Egypt kits and were expressed as nM/mg tissue protein [20].

2.3.4 Histopathological study of sciatic nerve

Half of samples were fixed in a mixture of 2.5% glutaraldehyde and 2.5% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 for 24 hours at 4°C. Tissue samples were further processed for E/M study. Longitudinal and transverse semi-thin sections were stained with toludin blue and examined under light microscope. Ultrathin sections were stained with lead citrate and uranyl acetate [21] and examined at Tanta Electron Microscope Unit.

2.4 Statistical Analysis

Data were expressed as mean ± standard error of mean (SEM) and analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test. The statistical evaluation of the data was performed using Graph Pad Prism 4.03 (Graph Pad Software, San Diego, California, USA). Values of $p< 0.05$ were considered statistically significant.

3. RESULTS

3.1 Behavioral Results

3.1.1 Tail flick test

As shown in Fig. 1; the diabetic untreated group showed significant ($P<0.05$) decrease in the latency time taken by the tail under the photoelectric cell (6.52±0.35 vs 12.45±0.19 sec) when compared with normal control group. However, treatment with insulin alone or calcitriol alone failed to increase the latency time (7.55±0.29, 6.90±0.17 vs 6.52±0.35 sec) respectively compared to the diabetic untreated group ($P >0.05$). Treatment with both insulin and calcitriol showed significant ($P<0.05$) increase in the latency time (8.11±0.32 vs 6.52±0.35 sec) as compared to diabetic untreated group although it was significantly impaired compared to control group (8.11±0.32 vs12.45±0.19 sec; $P<0.05$).

3.2 Biochemical Analysis

3.2.1 Blood glucose level

At the end of the experiment, the diabetic untreated rats had significant ($P<0.05$) increase in the blood glucose levels compared with the normal control group (423.64±4.4 vs 116.92±0.80 mg/dl).Treatment of the diabetic rats with either insulin or calcitriol alone or co-administration of their combinations resulted in a significant ($P<0.05$) decrease in the blood glucose level compared to diabetic untreated group (189.72±3.97; 274.53±4.17 and 152.02±4.29), respectively, with more significant reduction in blood glucose level in the co-administration of insulin and calcitriol as compared to either calcitriol or insulin treated group alone (Table 1).

3.2.2 Insulin level

As shown in Table 1; serum insulin level showed significant ($p < 0.05$) decrease in the diabetic untreated group, when compared with the normal control group (0.10±0.012 vs 2.82±0.088 ng/ml). Administration of insulin alone produced non-significant ($p>0.05$) changes compared with the diabetic untreated group (0.11±0.016 vs 0.10±0.012 ng/ml), while administration of calcitriol alone or combined with insulin produced significant ($p<0.05$) increase as compared with the diabetic untreated group (0.95±0.056 and1.04±0.058) respectively vs (0.10±0.012 ng/ml). However, treatment with calcitriol alone or with insulin significantly ($p<0.05$) increased serum insulin level compared to insulin treated group (0.95±0.056 and 1.04±0.058) respectively vs (0.11±0.016 ng/ml). On the other hand, co-administration of calcitriol and insulin did not show any significant difference compared with calcitriol treated group ($p>0.05$).

3.2.3 Advanced glycation end products (AGEs)

Table 1 shows a significant increase in serum level of AGEs ($p<0.05$) within diabetic untreated group compared with normal control group (249.52±2.21 vs 75.98±0.28 ng/l). Diabetic rats treated with either insulin or calcitriol or both insulin and calcitriol showed significant ($p<0.05$) decrease in AGES level compared with the diabetic untreated group (175.50±2.37; 153.74±1.68 and 86.65±2.03, respectively vs 249.52±2.21 ng/l). However, the level of AGEs was highly reduced to (86.65±2.03 ng/l) with co-

administration of calcitriol and insulin which was significant (p<0.05) compared with insulin treated group (175.50±2.37 ng/l) or calcitriol treated group (153.74±1.68 ng/l).

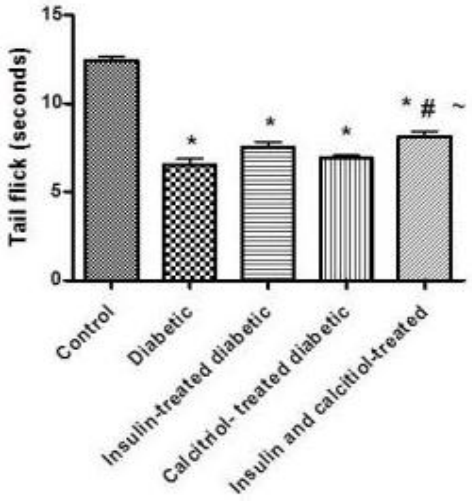

Fig. 1. Effect of calcitriol with or without insulin on the pain threshold values in streptozotocin-injected diabetic rats subjected to tail flick

**P< 0.05 as compared to normal control group; #P< 0.05 as compared to diabetic untreated group; †P< 0.05 as compared to insulin-treated diabetic group; ~P< 0.05 as compared to calcitriol-treated group; Number of rats in each group = 8 rats*

3.2.4 Superoxide dismutase (SOD) in sciatic nerve homogenate

Table 2 shows that SOD level decreased significantly (P<0.05) in diabetic untreated group compared with normal control group (9.15±0.19 vs 33.85±0.15 unit/mg tissue protein). Further, treatment with insulin alone or calcitriol alone or co-administration of insulin and calcitriol showed significant (P<0.05) increase compared with diabetic untreated group (13.62±0.18; 19.70±0.37 and 25.48±0.57 unit/mg tissue protein), respectively vs (9.15±0.19 unit/mg tissue protein). Also, treatment with calcitrol alone or co-administration with insulin significantly (p<0.05) increased level of SOD compared with insulin treated group (19.70±0.37 and 25.48±0.57), respectively vs (13.62±0.18) unit/mg tissue protein. Co-admistration of calcitriol and insulin significantly (p<0.05) increased level of SOD compared with calcitriol treated group (25.48±0.57 vs 19.70±0.37 unit/mg tissue).

3.2.5 Malondialdhye (MDA) in sciatic nerve homogenate

Table 2 shows that MDA level was significantly (P<0.05) increased in diabetic untreated group compared with normal control group (19.78±0.52 vs 4.91±0.17 nM/mg tissue protein).Treatment with insulin alone showed non- significant (P>0.05) changes compared with diabetic untreated group (17.77±0.48 vs19.78±0.52 nM/mg tissue protein). On the other hand, treatment with calcitriol alone or combined with insulin showed significant (P<0.05) decrease compared with diabetic untreated group (14.98±0.37 and 12.21±0.25), respectively vs (19.78±0.520 nM/mg tissue protein. Finally, as shown in Table 2, co-adminstration of calcitriol and insulin significantly (p<0.05) reduced level of MDA compared with insulin treated group (12.21±0.25 vs 17.77±0.48 nM/mg tissue protein) with no significant (p>0.05) difference between administration of calcitriol alone or co-administration with insulin.

3.2.6 Tumor necrosis factor alpha (TNF-α) in sciatic nerve homogenate

As shown in Table 2; TNF-α level was significantly (P<0.05) increased in diabetic untreated group compared to normal control group (141.20±1.84 vs 30.36±0.24 pg/ml).Treatment of diabetic rats with insulin alone, calcitriol alone or both showed significant (P<0.05) decrease in the TNF-α compared to diabetic untreated group (125.75±0.92; 92.97±1.08 and 64.37±1.63 pg/ml) respectively vs (141.20±1.84 pg/ml). Also, treatment with calcitrol alone or co-administration with insulin significantly (p<0.05) decreased level of TNF-α compared with insulin treated group (92.97±1.08 and 64.37±1.63), respectively vs (125.75±0.92 pg/ml). Moreover, calcitriol and insulin co-administration significantly (p<0.05) decreased the level of TNF-α compared with calcitriol treated group (64.37±1.63 vs 92.97±1.08 pg/ml).

3.3 Histological Results

3.3.1 Light microscopic results

1-Normal control group: Sections of this group stained with toluidine blue showed densely packed groups of different sized, mylinated and unmylinated nerve fibers that enclosed within

Table 1. Effect of calcitriol with or without insulin on blood glucose level before and after induction of diabetes, serum insulin and serum advanced glycation end products (AGEs) of diabetic rats

	Normal control	Diabetic untreated	Insulin-treated diabetic	Calcitriol-treated diabetic	insulin and calcitriol- treated diabetic
Blood glucose at start of experiment (mg/dl)	98.63±2.28	94.50±3.63	100.38±4.84	93.75±4.39	97.37±4.86
Blood glucose after STZ injection (mg/dl)	118.38±0.80	397.62±20.23*	432.12±13.02*	404.00±6.63*	433.50±17.92*
Blood glucose after 4 weeks of treatment (mg/dl)	116.92±0.80	423.64±4.42*	189.72±3.97$^{*\#}$	274.53±4.17$^{*\#\dagger}$	152.02±4.29$^{*\#\sim\dagger}$
Blood insulin (ng/ml)	2.82±0.088	0.10±0.012*	0.11±0.016*	0.95±0.056$^{*\#\dagger}$	1.04±0.058$^{*\#\dagger}$
(AGEs) (ng/l)	75.98±0.28	249.52±2.21*	175.50±2.37$^{*\#}$	153.74±1.68$^{*\#\dagger}$	86.65±2.03$^{*\#\sim\dagger}$

Data expressed as mean ± SEM, Number of rats in each group =8 rats, $^{*}P< 0.05$ *as compared to normal control group;* $^{\#}P< 0.05$ *as compared to diabetic untreated group;* $^{\dagger}P< 0.05$ *as compared to insulin-treated diabetic group;* $^{\sim}P< 0.05$ *as compared to calcitriol-treated group*

Table 2. Effect of calcitriol with or without insulin on superoxide dismutase (SOD), malondialdhyde (MDA) and tumor necrosis factor alpha (TNF-α) in the sciatic nerve homogenate of diabetic rats

	Normal control	Diabetic untreated	Insulin- treated diabetic	Calcitriol-treated diabetic	insulin and calcitriol- treated diabetic
SOD(u/mg tissue protein)	33.85±0.15	9.15±0.19*	13.62±0.18$^{*\#}$	19.70±0.37$^{*\#\dagger}$	25.48±0.57$^{*\#\sim\dagger}$
MDA(nM/mg tissue protein)	4.91±0.17	19.78±0.52*	17.77±0.48*	14.98±0.37$^{*\#}$	12.21±0.25$^{*\#\dagger}$
TNF-α (pg/ml)	30.36±0.24	141.20±1.84*	125.75±0.92$^{*\#}$	92.97±1.08$^{*\#\dagger}$	64.37±1.63$^{*\#\sim\dagger}$

Data expressed as mean ± SEM, Number of rats in each group =8 rats, $^{*}P< 0.05$ *as compared to control group;* $^{\#}P< 0.05$ *as compared to diabetic untreated group; ,* $^{\dagger}P< 0.05$ *as compared to insulin-treated diabetic group; ,* $^{\sim}P< 0.05$ *as compared to calcitriol-treated group*

endoneurium and surrounded by perineurium. The whole trunk was surrounded by CT epineurium. The mylinated nerve fibers revealed regular compact myelin with minimal infoldings. Schwann cells and fibroblasts were seen lodged between nerve fibers (Plate. 1-A&B).

2-Untreated diabetic group: Sections of this group revealed thickening and irregularity of the nerve fibers in the form of evagination, invagination and splitting (Plate. 1C) and appearance of bubbles (Plate 1 D). Fibroblasts and mast cells were encountered among connective tissue endoneurium (Plate 1 D).The nerve fibers were widely separated from each other with appearance of tissue ruminants (Plate 1 C).

3-Insulin-treated- diabetic group: Sections of this group revealed mild improvement; most of myelinated nerve fibers showed infoldings and heterogeneity of myelin with appearance of bubbles. However, few mylinated fibers appeared with mild infoldiong. (Plate 2- A&B).

4-Calcitrol treated diabetic group: Sections of this group showed moderate improvement; some of myelinated nerve fibers appeared with minimal infolding and with compact, uniform thickness. However, other fibers showed irregular thickening and infolding (Plate 2-C&D).

5-Insulin- calcitrol-treated- diabetic group: Sections of this group revealed good improvement; the majority of myelinated nerve

fibers were of nearly normal compact appearance and with uniform thickening. Other few nerve fibers showed minimal infoldings (Plate 2- E&F).

3.3.2 Electronic microscopic results

1-Normal control group: The myelin sheath of mylinated nerve fibers was compact and of uniform thickness. The axoplasm contained microtubules, micro-filaments and membrane-bound vesicles. The unmylinated nerve fibers were circular or ovoid in shape and grouped in clusters or occupied deep recesses on the surface of Schwann cell (Plate 3-A). The Schwann cell cytoplasm wrapped around mylinated nerve fiber and housed large vesicular nucleus (Plate 3-B), rER, mitochondria (Plate 3-C). Collagen fibers were observed in the connective tissue endonurium (Plate 3-C&D).

2-Untreated Diabetic Group: Sections of this group revealed abnormal thickening and heterogeneity of myelin sheath together with appearance of myelin bubbles and whorled like structure (Plate 4-A&B). The evagination, invagination and splitting of myelin sheath (Plate 4- C&D) caused compression of the axoplasm to an apparent small irregular diameter. The axoplasm contained numerous vesicles and whorled like structure (Plate 4-E). Fibroblast and collagen fibers were aggregated in the CT endoneurium (Plate 4-A&D). Some unmylinated axons appeared with irregular outer contour (Plate 4-F) andthe axoplasm showed electron dense bodies and vesicles (Plate 4-F&G).

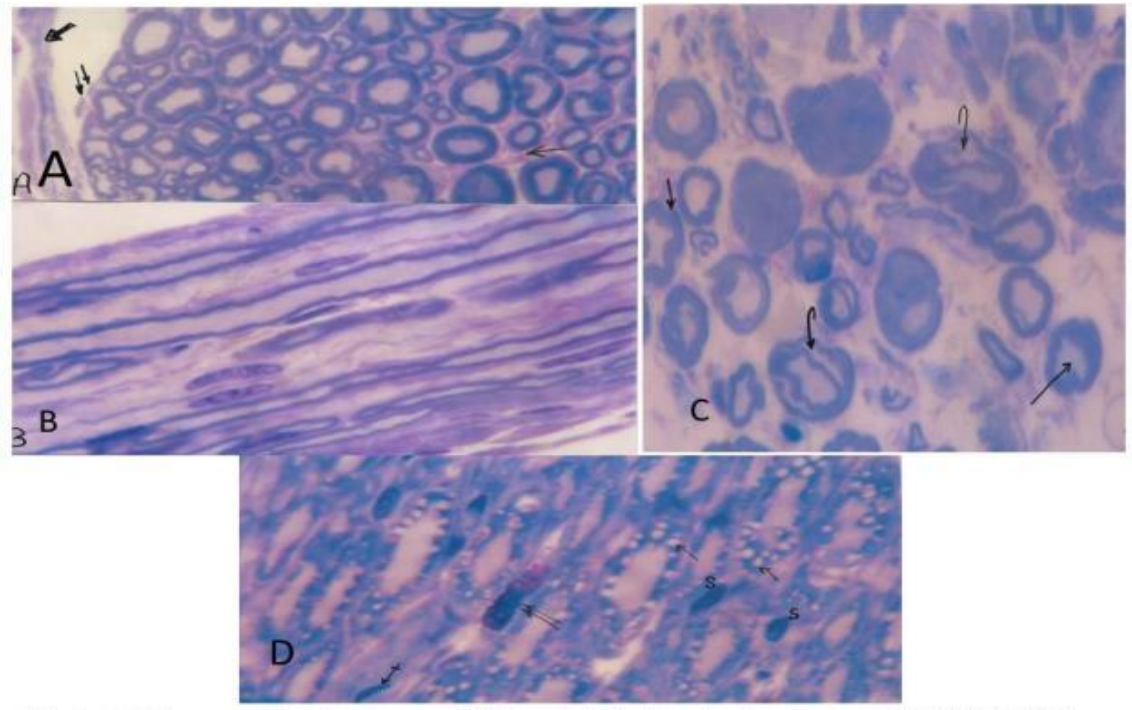

Plate 1. Sciatic nerve of control group (A&B) and diabetic untreated group (C&D) (TB X1000)

1-A: A photomicrograph of sciatic nerve of control group showing transverse sections of densely packed mylinated and unmylinated nerve fibers. Notice CT endoneurium (thin arrow), CT perinurium (double arrows) and CT epineurium (thick arrow) (T B X1000)

1-B: A photomicrograph of sciatic nerve of control group showing longitudinal sections of densely packed nerve fibers. Notice Schwann cell (S) and fibroblast (arrow) in the endoneurium. (T B X 1000)

1-C: A photomicrograph of sciatic nerve of diabetic- untreated group showing thickening and irregularity of mylinated nerve fibers in the form of invagination of myelin sheath (long arrow), evagination (short arrow) and splitting of myelin sheath (curved arrow) (T B X 1000)

1-D: A photomicrograph of sciatic nerve of diabetic- untreated group showing mylinated nerve fibers with appearance of bubbles in their myelin (arrow). Notice mast cell (double arrows), Schwann cell (S) and fibroblast (crossed arrow) in CT endonurium (TB X1000)

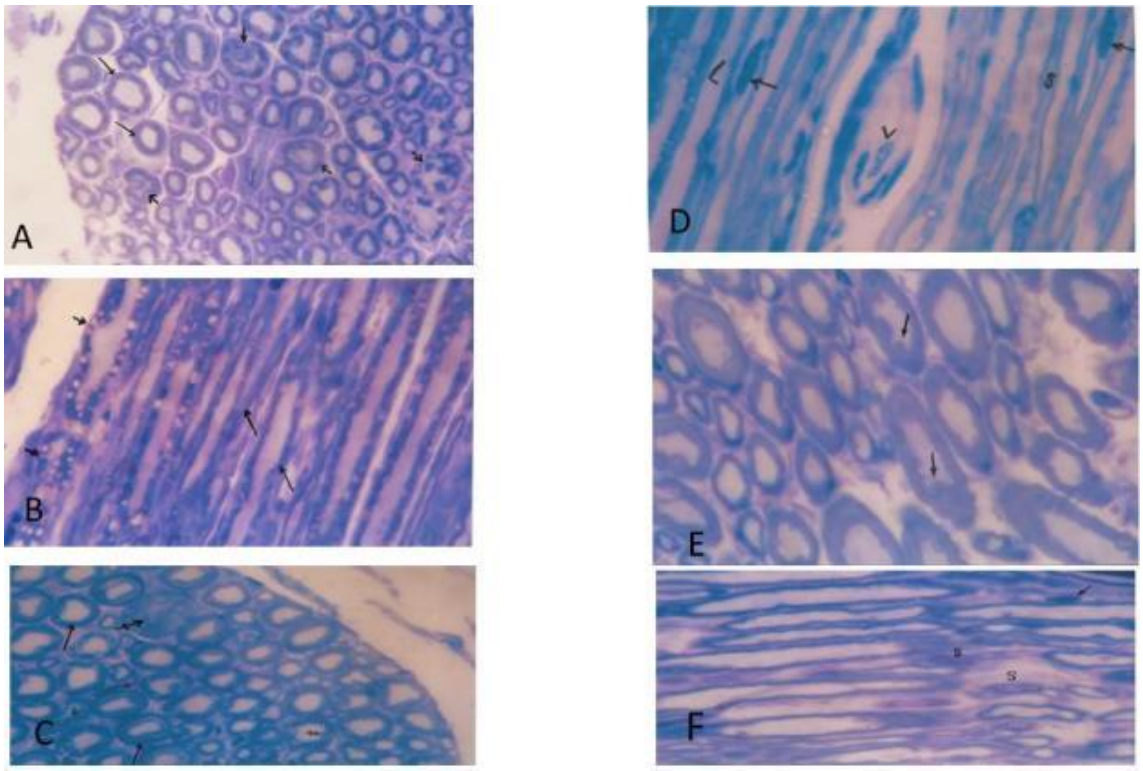

Plate 2. Sciatic nerve of insulin-treated diabetic group (A&B), calcitriol- treated(C&D) and insulin-calcitriol treated group (E&F) (TB X1000)

2-A: A photomicrograph of transverse section of sciatic nerve of insulin-treated diabetic group showing most of mylinated nerve fibers with infolding and heterogeneity of their myelin sheath (short arrow). Other few fibers show minimal infolding (long arrow) (T B X1000)

2-B: A photomicrograph of Long sections of sciatic nerve of insulin-treated diabetic group showing some fibers with heterogeneity of their myelin sheath and appearance of bubbles (short arrow). Other few fibers show minimal infolding (long arrow) (T B X1000)

2-C: A Photomicrograph of transverse sections of sciatic nerve of calcitrol-treated diabetic group showing some fibers with normal uniform thickening and with minimal infolding (long arrow). Other fibers show heterogeneity of their myelin sheath with major infolding (crossed arrow). Notice fibroblast in the CT endoneurium (short arrow) and blood vessel (V).(TB X1000)

2-D: A Photomicrograph of long section of sciatic nerve of calcitrol-treated diabetic group showing some fibers with normal uniform thickening and with minimal infolding (N). Other fibers show heterogeneity of their myelin sheath with appearance of bubbles (curved arrow). Notice Schwann cell (arrow) and blood vessel (V)(TB X1000)

2-E: A photomicrograph of transverse section of sciatic nerve of insulin- calcitrol- treated diabetic group showing mylinated fibers with normal uniform thickening. Other few fibers show infolding of myelin sheath (arrow) (TB X 1000)

2-F: A photomicrograph of long. sections of sciatic nerve of insulin- calcitrol-treated diabetic group showing most of mylinated fibers with normal uniform thickening. Notice Schwann cell (S) and fibroblast (arrow) among nerve fibers (TB X 1000)

3-Insulin- treated Diabetic group: Sections of this group showed mild improvement; some fibers appeared with minimal infolding and of uniform thickening like control (Plate 5-A), however most of fibers showed irregular thickening, evagination, invagination of their myelin sheath and compression of their axoplasm (Plate 5- A&B). Numerous collagen fibers and fibroblasts, which extended their processes among the nerve fibers, were observed in the CT endoneurium (Plate 5-B).

4-Calcitriol- Treated Diabetic Group: Sections of this group revealed moderate improvement; some of mylinated nerve fibers appeared with regular uniform thickening of their myelin with minor infoldings. However, other mylinated nerve fibers showed electron dense bodies in their

axoplasm and invagination (Plate 5-C). Fibroblasts and numerous collagen fibers were still encountered in CT endonurium (Plate 5-D).

5-Insulin-Calcitriol- treated Diabetic Group: Sections of this group showed good improvement of ultrastructure. The myelinated nerve fibers appeared with uniform thickness and with regular contour. The axoplasm contained microvesicles and microtubules; however, some contained electron dense bodies. The nerve fibers were packed within CT endoneurium. The unmylinated nerve fibers appeared in groups (Plate 5- E&F).

4. DISCUSSION

Diabetic neuropathy is one of the major complications of DM that include diabetic nephropathy, diabetic retinopathy and diabetic cardiomyopathy. In human there is an association between DPN and vitamin D deficiency [22]. It is interesting to highlight the link between vitamin D and cognition which suggests a direct impact of vitamin D on nerve function [23]. DN is associated with decrease in pain threshold that can be examined by behavioral nociceptive tests like painful neuropathic pain models as thermal hyperalgesia.

In the present study, DPN was detected experimentally by behavioral test of hyperalgesia measured by tail flick apparatus. The diabetic group showed significant decrease in the time taken under the photoelectric cell, which means that the diabetic untreated group showed decrease in the pain threshold compared with

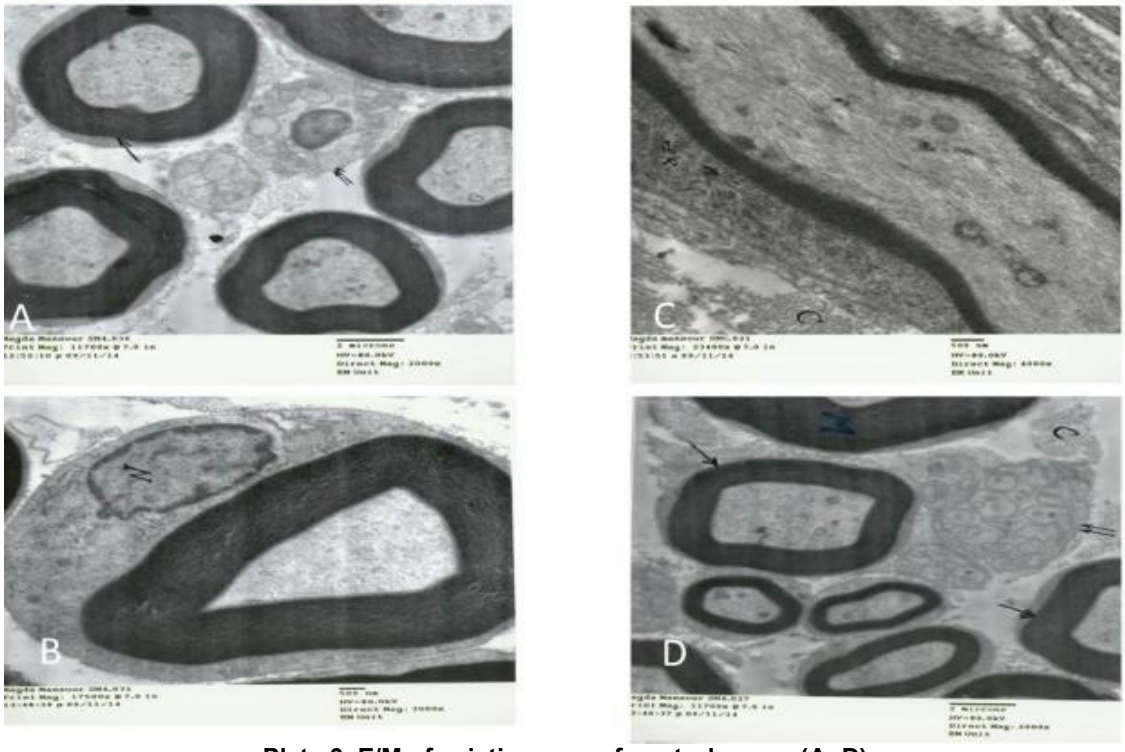

Plate 3. E/M of sciatic nerve of control group (A- D)

***3-A**: Electron micrograph of control group sciatic nerve showing group of myelinated nerve fibers with intact regular myelin sheath (arrow) and unmylinated nerve fibers grouped in clusters or occupied deep recesses on Schwann cell surface (double arrows) (X2000)*

***3-B**: Electron micrograph of control group sciatic nerve showing one myelinated nerve fiber with intact regular myelin sheath, surrounded by Schwann cell housing large vesicular nucleus (N). (X3000)*

***3-C**: Electron micrograph of LS of control group sciatic nerve showing one myelinated nerve fiber with intact regular myelin sheath, surrounded by Schwann cell cytoplasm housing rER and mitochondria (M). Notice TS of collagen fibers (c) (X4000)*

***3-D**: Electron micrograph of control group sciatic nerve showing group of myelinated nerve fibers with intact regular myelin sheath (arrow) and unmylinated nerve fibers grouped in clusters (double arrows). Notice TS of collagen fibers in the CT endonurium (C). (X2000)*

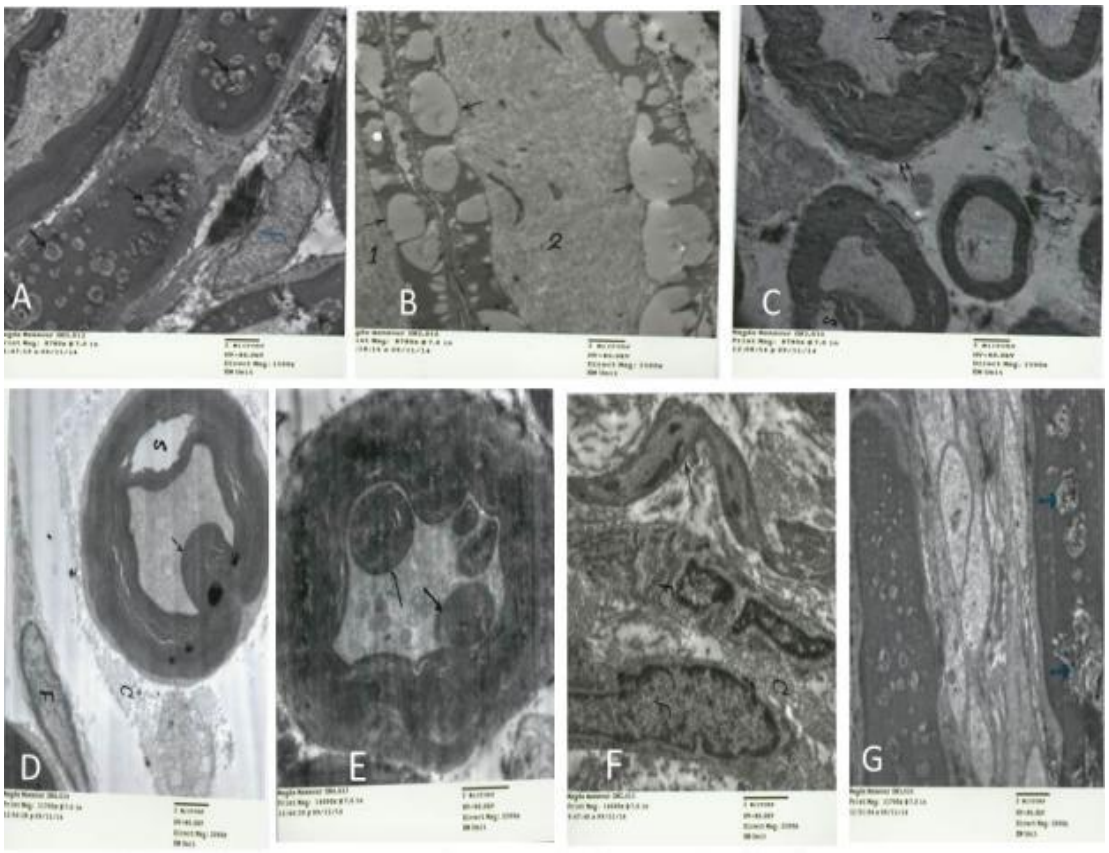

Plate 4. E/M of sciatic nerve of untreated diabetic group (A- G)

***4-A**: Electron micrograph of sciatic nerve of diabetic untreated group showingabnormal thickeningand heterogeneity of myelin sheath with appearance of whorled- like structures filled with heterogeneous substance (arrow). Notice fibroblast (F) in the CT endoneurium (X 1500)*

***4-B**: Electron micrograph of sciatic nerve of diabetic untreated group showing2 adjacent myelinated nerve fibers (1&2) having abnormal myelin sheath with numerous bubbles (arrows) (X 1500)*

***4-C**: Electron micrograph of sciatic nerve of diabetic untreated group showing invagination (inward pulging, arrow), evagination (outward pulging, double arrows) and splitting (S) of myelin sheath causing compression the axoplasm (X1500)*

***4-D**: Electron micrograph of sciatic nerve of diabetic untreated group showing invagination (arrow) and splitting (S) of myelin sheath. Notice group of unmylinated nerve fibers, fibroblast (F) and collagen (C). (X2000)*

***4-E**: Electron micrograph of sciatic nerve of diabetic untreated group showing abnormal thickening and invaginationof myelin sheath causing compression of the axoplasm which is filled with abnormal whorled like structure (arrow) (X2500)*

***4-F**: Electron micrograph of sciatic nerve of diabetic untreated group showing active fibroblasts (F), numerous collagen fibers (C) in CT endonurium and abnormal unmylinated nerve fiber (arrow) (2500)*

***4-G**: Electron micrograph of sciatic nerve of diabetic untreated group showing one mylinated nerve fiber (upper) with abnormal myelin sheath filled with whorled like structure. Another mylinated nerve fiber (lower) shows various forms of whorled like structures. Group of unmylinated nerve fibers (un) having electron dense bodies in their axoplasm are also seen in the middle (X2000)*

normal control group. These results are in agreement with Han et al. [24,25] and Huang et al. [24,25]. The hyperalgesic effect was reversed only by co-administration of insulin and calcitriol.

This could be explained on the basis of the effect of calcitrol that improves endogenous insulin release through its direct effect on beta cells, thus increasing insulin secretion by activation of vitamin D receptors (VDR) of pancreatic beta cells. It also promotes beta- cell survival by modulating the effect of cytokines on pancreatic tissue [11].

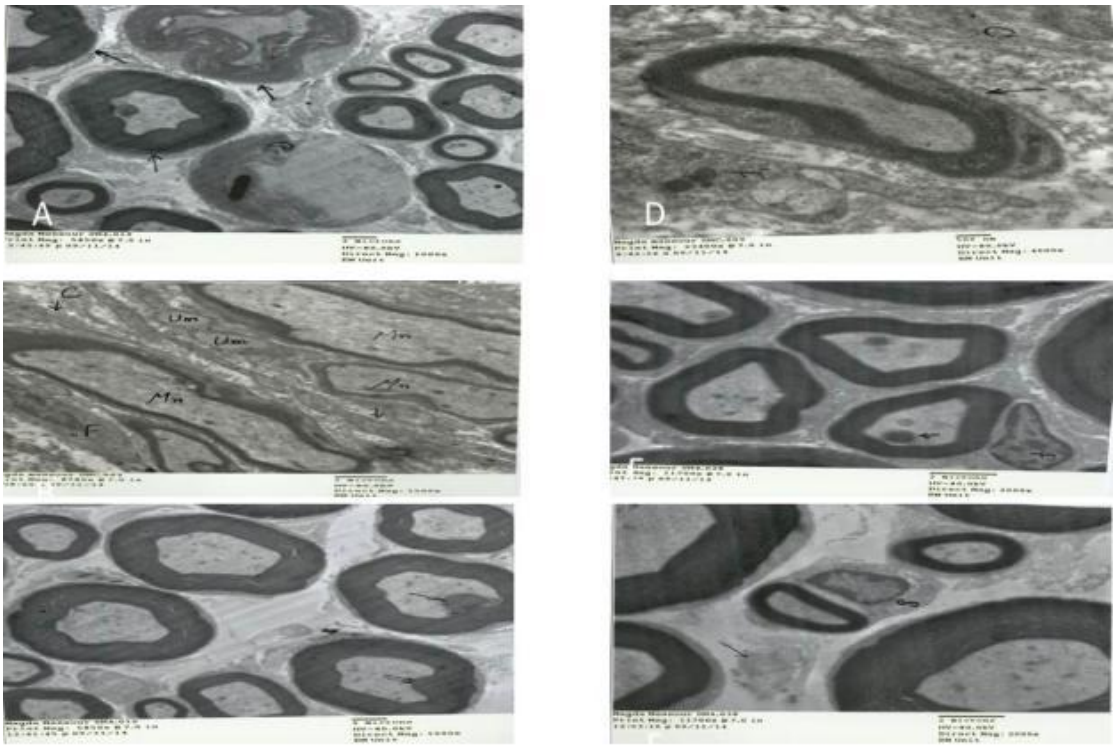

Plate 5. E/M of sciatic nerve of insulin treated diabetic group (A&B), calcitriol treated diabetic group (C&D) and insulin- calcitriol treated diabetic group (E&F)

***5-A**: Electron micrograph of sciatic nerve of insulin- treated diabetic group showing some normal mylinated fibers with minimal infolding (N), while most of fibers appear with abnormal thickening and major infolding (arrow) (X1000)*

***5-B**: Electron micrograph of sciatic nerve of insulin- treated diabetic group showing abnormal thickening of mylinated fibers (Mn). Notice numerous fibroblasts (F) with their processes (arrow) extending in between nerve fibers and collagen fibers (c). Some unmylinated fibers (Um) are also seen (X 1500)*

***5-C**: Electron micrograph of sciatic nerve of calcitriol- treated diabetic group showing most of the fibers with uniform normal thickening of their myelin sheath. Few fibers appear with electron dense bodies in their axoplasm (arrow), other appears with invagination (double arrows) (X 1000)*

***5-D**: Electron micrograph of sciatic nerve of calcitriol- treated diabetic group showing numerous collagen fibers (C) and fibroblast (F) in the CT endonurium around mylinated nerve fiber (arrow). (X4000)*

***5-E**: Electron micrograph of sciatic nerve of insulin-calcitriol- treated diabetic group showing nearly normal mylinated nerve fibers with regular compact myelin sheath. The axoplasm of some fibers shows electron dense bodies (arrow). Notice the fibroblast (F) in the densly packed CT endonurium. (X 2000)*

***5-F:** Electron micrograph of sciatic nerve of insulin-calcitriol- treated diabetic group showing nearly normal mylinated nerve fibers with regular compact myelin sheath. One fiber shows Schwann cell (S) around its myelin sheath. Group of unmylinated nerve fibers is also seen (X 2000)*

With reference to AGEs, the present study showed a significant increase in AGEs level in diabetic group compared with normal control one. These results are in line with Wei et al. [26] who postulated that male Wistar rats fed on high fat diet for eight weeks then injected with a small dose of STZ (25 mg/kg) induced T2DM with insulin resistance and an increase in AGEs. Treatment with calcitriol alone or combined with insulin showed significant decrease in the AGEs compared with diabetic untreated group. These results agree with Lee et al. [15] who postulated that calcitriol modulates the AGEs production by binding to its receptors. The accumulation of proteins damaged by formation of AGEs residues in the extracellular matrix (ECM) of the peripheral nerves in DM had been linked to changes in nerve structure and neuronal function and development of diabetic neuropathy [27]. Direct glycation of ECM proteins particularly the laminin and fibronectin ingredients of the endoneurium of rat sciatic nerves of STZ-induced DM decreased

the ability of sensory neurons to extend neuritis with failure of regeneration after injuries [28]. Calcitriol protects the peripheral nerves from the damaging effects of AGEs, where their role in DN is expressed through receptors for advanced glycation end products (RAGE) that founded in the endothelial and Schwann cells of perineural and endoneural vessels. The modification of proteins with AGEs causes structural and functional alterations in the peripheral nerves through induction of neural and Schwann cell death [29].

In the present study, sciatic nerve homogenate showed significant decrease in superoxide dismutase (SOD), which was exhausted due to overproduction of ROS induced by hyperglycemia. These result are in parallel with Wu et al. [30]. On the other hand, MDA which is a marker of lipid peroxidation showed significant increase in the diabetic untreated rats, which run with the progression of the disease as a result of destruction of lipid membrane via rearrangement of the double bond in the unsaturated fatty acids of the membrane caused tissue damage. The enhanced lipid peroxidation provides an index of oxidative stress occurred during DM. Because of the increase in the oxidants and the decrease in total antioxidants, the oxidative/antioxidative balance demonstrated to shift towards the oxidative status in brain and sciatic nerve. These results are in agreement with Alp et al. [31]. Likewise, the diabetic untreated group showed increase of the TNF-α, which is a key marker for inflammatory condition associated with DM and it is responsible for tissue necrosis. Therefore, we could postulate that the metabolic dysfunction associated with high fat diet and insulin resistance in T2DM may cause reduced neurotrophic support and reduced regeneration of the distal axon. Besides, the loss of appropriate insulin signaling could make neurons even more susceptible to these inflammatory pathogenic cascades in diabetic untreated group compared with the normal control group. These results are in accordance with Sugimoto et al. [32] who concluded that a low dose of insulin, insufficient to affect systemic glycemia, partially restored the impaired peripheral nerve insulin receptor signaling and ameliorated peripheral sensory nerve dysfunction in STZ-induced diabetic rats. These findings support the notion that, besides hyperglycemia, insulin deficiency is involved in the pathogenesis of diabetes.

Cotter and Cameron [33] suggested that the imbalance between formation of free radicals and the endogenous antioxidant enzyme resulted in the generation of oxidative stress. Within the neuron, the SOD enzyme that is responsible for conversion of superoxide anions to H2O2 regulated the redox balance. Consequently oxidative stress that cause exhaustion of SOD lead to activation of aldose reductase and protein kinase C due to elevated levels of superoxide anions in neurons, thus resulting in elevated pain perception in the neurons [34]. These free radical bind with superoxide anion to form peroxynitrite, which rapidly causes protein nitration or nitrosylation, lipid peroxidation, DNA damage, cell death and had direct toxic effects on the nerve tissue leading to neuropathic pain [35,36]. Kumar et al. [37] suggested that hyperglycemia leads to reduced threshold of pain due to increased oxidative stress, AGEs and inflammatory mediators.

Insulin treatment increased SOD and decreased TNF-α, but there were no significant change in MDA when compared with diabetic untreated group. These results could be due to the exogenous insulin which may play a more important role in lipid and glucose metabolism, gene regulation or mitochondrial maintenance in peripheral neurons but it has no antioxidant activity that could protect the peripheral neurons from the oxidative stress resulted during DM. These results agree with Ozaki et al. [38] who suggested that there was no significant amelioration of microangiopathy by insulin treatment despite of the decrease of peripheral neuropathic changes. Co-administration of calcitriol with insulin, in the present study, caused significant reduction of TNF-α, MDA and increase in SOD. This is in partial agreement with previous studies, which suggested that insulin alone partially, reversed the neuropathic pain in STZ induced diabetic rats. On the other hand, insulin with antioxidant agent combination not only attenuated the diabetic condition but also reversed neuropathic pain through modulation of oxidative–nitrosative stress, inflammatory cytokine release and reduction of apoptosis in the STZ induced diabetic rats [35,39].

All these behavioral and biochemical investigations done on the sciatic nerve homogenate were supported by histological examination of sciatic nerve that revealed some histological changes in myelinated axons. These changes in diabetic untreated group were in the form of invagination, evagination and focal thickening of myelin sheath, in addition to the

heterogeneity of the myelin structure with appearance of myelin bubbles and splitting of its layers in some parts. These changes in myelin sheath resulted in compression of the axoplasm in some parts. Also, unmyelinated nerve fibers showed abnormal contour and presence of electron dense bodies, while other fibers were lost leaving fiber remnants and debris. These changes could be attributed to the oxidative stress generated by free radicals which impair the blood supply to the neurons and leading to impaired neuronal function and hypoxia [40]. Kim et al. [36] attributed these changes to the elevated level of nitric oxide (NO) in diabetic rat. They reported that the elevated level of NO leads to formation of peroxynitrate which cause lipid peroxidation, DNA damage and cell death which intern leads to neuropathic pain. Shi et al. [41] attributed these changes in DPN to demyelination of nerve fibers, disorganization of lamellar and axonal structures and decreased expression of myelin basic proteins in the nerve tissue. The presence of fibroblasts and excess collagen in histological pictures of diabetic rat of this study could be a reflection of the degenerative effect of diabetes on peripheral nerves. Schwann cells could influence adjacent fibroblasts, thus promoting synthesis of collagen fibrils. The presence of macrophages and mast cells as members of inflammatory cells in the sciatic nerve of diabetic rat of this study was in agreement with evidence from both animal and human studies, which indicate that systemic inflammation is involved in the pathophysiological process of DM. These findings go hand in hand with the elevated level of TNF-α, an inflammatory cytokine in diabetic group of the present study. The same finding was reported by Wang et al. who added that TNF-α initiate the release of other inflammatory cytokines including IL-1B and IL-2 that are responsible for causing neuropathic pain [42].

Regarding the noted mild improvement in sciatic nerve morphology in diabetic group treated with insulin, these results are in accordance with Ozaki et al. who postulated that insulin treatment inhibited endoneural fibrosis, but not the vascular lesions [38]. However, moderate improvement was reported in diabetic group treated with calcitriol alone. But, good amelioration and improvement was reported in concomitant administration of insulin and calcitriol for diabetic rats. These results could be attributed to the cytoprotective effect of antioxidant vitamin D3 on the peripheral nerves.

5. CONCLUSION

Our study shows that calcitriol improved physiologic symptoms of neuropathy in diabetic rats. Also, it should be mentioned that co-administration of calcitriol with insulin has the most positive effects on some indices of diabetic neuropathy. Results of the current study suggest that calcitriol could have a potential therapeutic role in diabetic neuropathy.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the appropriate our local ethics committee Faculty of Medicine, Menoufia University.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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